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II. THE BIURET REACTION OF DI-ACID AMIDES.

By MARY M. RISING, JOSEPH S. HICKS, AND GEORGINE
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(From the Kent Chemical Laboratory of the University of Chicago, Chicago.)

There was published recently[1] in this *Journal* a paper describing a study of the biuret reaction of acid imides. The work discussed therein constitutes an early part of an extensive investigation of the chemistry of the biuret reaction, undertaken for the purpose of ascertaining (1) the chemical nature and molecular structure of the colored products formed in the reaction of acid imides, di-acid amides, amino acid amides, and polypeptides with the biuret reagents cupric ion and alkali, and (2) the atoms or groups of atoms in molecules which show the biuret reaction which are essential for the occurrence of the reaction. It is hoped that the results of these investigations will uncover clues relating to the molecular structure of the proteins, which so characteristically form colored products with the biuret reagents.

The present paper discusses the biuret reaction of di-acid amides. The behavior of malonamide and certain of its N-alkyl and N-aryl derivatives, and of oxamide and some alkylated oxamides, with cupric ion and alkali is described, the isolation of the sodium copper salts of biuret, malonamide, monoethyloxamide, and symmetrical diethyloxamide is reported, and structures for these salts are proposed, based upon analytical data and the behavior of the salts. In addition, the theory of the biuret reaction, the development of

[1] Rising, M. M., and Johnson, C. A., *J. Biol. Chem.*, 80, 709 (1928).

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which was begun by Schiff,[2] and continued in our earlier paper, is augmented. Suggestions are made to explain certain phenomena observed in connection with the reactions of the amides studied, such as the well defined difference in the behavior of di-acid amides and acid imides towards the biuret reagents, and the failure of N-polysubstituted di-acid amides to show the biuret reaction. Suggestions are made further as to the atoms in di-acid amide molecules which take part in the biuret reaction, and with regard to conditions which are favorable for the occurrence of the reaction.

The logical point of departure for a study of the biuret reaction of di-acid amides is the biuret reaction of biuret, $NH_2 \cdot CO \cdot NH \cdot CO \cdot NH_2$, itself a di-acid amide. This beginning was made by Schiff in his isolation of potassium copper biuret, the formula of which he proved to be $K_2Cu \, (biuret)_2 \cdot 4 \, H_2O$. Similarly, the sodium copper salt of malonamide, $NH_2 \cdot CO \cdot CH_2 \cdot CO \cdot NH_2$, which is closely related to biuret, of monoethyloxamide, $C_2H_5NH \cdot CO \cdot CO \cdot NH_2$, and of symmetrical diethyloxamide, $C_2H_5NH \cdot CO \cdot CO \cdot NHC_2H_5$, all isolated by us, have been found to have the formula $Me_2Cu \, (di\text{-}acid \, amide)_2 \cdot xH_2O$. An inspection of the structures of the amides from which the salts are derived makes it apparent that di-acid amides of a variety of structural types react with the biuret reagents to produce salts containing a ratio of 2 atoms of alkali metal to 1 atom of copper to 2 molecules of amide. Schiff himself distinguished three types of di-acid amides showing the biuret reaction: (1) those with the group $NH_2 \cdot \underset{\|}{C} \cdot \underset{|}{N} \cdot \underset{\|}{C} \cdot NH_2$, (2) those with the group $NH_2 \cdot \underset{\|}{C} \cdot \underset{|}{C} \cdot \underset{\|}{C} \cdot NH_2$, and (3) those with the group $NH_2 \cdot \underset{\|}{C} \cdot \underset{\|}{C} \cdot NH_2$. Biuret (Group 1), malonamide (Group 2), and oxamide (Group 3) all form alkali copper salts of the formula $Me_2Cu \, (di\text{-}acid \, amide)_2$, as we have now found, and it is undoubtedly significant from the point of view of the atoms in a di-acid amide molecule which are essential for the occurrence of the biuret reaction that di-acid amides with these marked structural variations form products of identical formula with alkali and cu-

[2] Schiff, H., *Ber. chem. Ges.*, **29**, 298 (1896); *Ann. Chem.*, **299**, 236 (1898); **319**, 287 (1901); **352**, 73 (1907).

pric ion. It is quite probable that the matter of atoms required for the occurrence of the reaction may be reduced to much simpler terms than was done by Schiff in his classification: it is altogether likely that the atoms requisite for a biuret reaction in the case of a di-acid amide are common to all three of Schiff's groups, and that if the atoms in these groups not concerned in the reaction are excluded, the three groups become identical. The Schiff classification has its chief value as an aid in predicting which di-acid amides will, and which will not, show the biuret reaction. It predicts, for example, the succinamide, $NH_2 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CO \cdot NH_2$, and hydrazodicarbonamide, $NH_2 \cdot CO \cdot NH \cdot NH \cdot CO \cdot NH_2$, will show no biuret reaction, since they are structurally unlike the three types mentioned; and it is indeed the case that these two di-acid amides show no biuret reaction under any conditions so far used for the test. While the Schiff classification takes into account the inhibition of the occurrence of the biuret reaction when the two acid amide groups in the molecule are separated by more than a single nitrogen atom or carbon atom (as in succinamide and hydrazodicarbonamide), an inhibition which we shall presently attempt to explain, it fails to recognize two other important aspects of the biuret reaction of di-acid amides: (1) Certain N-alkylated di-acid amides show the reaction, notably monoethyloxamide, with the group $RNH \cdot \underset{\parallel}{C} \cdot \underset{\parallel}{C} \cdot NH_2$, and symmetrical diethyloxamide with the group $RNH \cdot \underset{\parallel}{C} \cdot \underset{\parallel}{C} \cdot NHR$. These two groups, in common with Schiff's three, contain the atoms essential for the occurrence of the reaction, which are probably the same for all di-acid amides which react with the biuret reagents. (2) The multiple substitution of hydrogen atoms attached to nitrogen in the di-acid amide molecule by alkyl radicals may entirely prevent the occurrence of the biuret reaction, as will be shown in the present work. The Schiff classification includes only di-acid amides; he does not extend his theory to acid imides with the characteristic group $-\underset{\parallel}{C} \cdot NH \cdot \underset{\parallel}{C}-$; many imides form colored products with the biuret reagents, having the formula $Me_2Cu\ (imide)_4 \cdot xH_2O$.[1,3]

[3] Tschugaeff, L., *Ber. chem. Ges.*, **38**, 2899 (1905); **39**, 3190 (1906); **40**, 1973 (1907). Ley, H., and Werner, F., *Ber. chem. Ges.*, **38**, 2199 (1905); **40**, 705 (1907).

Nor does the Schiff theory include amino acid amides with the characteristic group $-\overset{\displaystyle |}{\underset{\displaystyle NH_2}{C}}-\overset{\displaystyle ||}{\underset{\displaystyle O}{C}}-NH_2$.   Products of the biuret reaction of amino acid amides are being studied by one of the present authors (Rising) in collaboration with Peter S. Yang.

In order to profit by the experience of Schiff, we began our work with the isolation of the product of the biuret reaction of biuret itself, sodium copper biuret.   The salt was obtained by treatment of biuret with 4 mols of sodium hydroxide and 1 mol of cupric acetate in water, and subsequent precipitation of it from the reaction

TABLE I.

Analytical Data for Sodium Copper Biuret.

Element.	Percentage composition.		Calculated percentage composition for $Na_2CuC_4H_{10}O_6N_6$ (mol. wt. 347.718).
	Sample I.	Sample II.	
Na	13.10	13.32	13.23
Cu	18.33	18.32	18.28
C	13.73	13.80	13.81
H	2.80	2.85	2.90
N	24.01	24.27	24.17
O			27.61
		.	100.00

mixture by means of the addition of 20 volumes of alcohol.   The reaction product was a rose-pink amorphous substance, quickly decomposed by water and all acids.   The analytical data for two samples of the product of a single preparation of sodium copper biuret are to be found in Table I.   Details regarding the procedure used to obtain this salt and others described are to be found in the experimental part of this paper.

The percentage composition of the salt agrees very well with that calculated for a molecule of empirical formula $Na_2CuC_4H_{10}O_6N_6$, and this formula corresponds closely to Me_2Cu (di-acid amide)$_2 \cdot xH_2O$ (biuret is $C_2H_5O_2N_3$, and $Na_2CuC_4H_{10}O_6N_6$ is $Na_2Cu(C_2H_3O_2N_3)_2 \cdot 2H_2O$).   At present no satisfactory data for the molecular weight of this salt and the others discussed in the present paper are available; it is therefore assumed tentatively that the

empirical formulas of the salts are also the molecular ones. With this assumption in mind a structure for the salt is proposed.
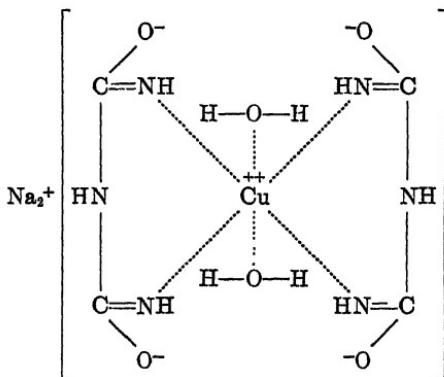
Sodium copper biuret.

This structure indicates that sodium hydroxide reacts with an acid tautomer of biuret to form a salt.[4] Salt formation increases the basicity of the biuret molecule which, acting as an amine, forms a complex with cupric ion. In this complex there is only a minute ionization of cupric ion, due perhaps to the restraining effect of the union of auxiliary valences of copper with those of nitrogen and oxygen.

It will be noted that the complex ion in sodium copper biuret carries an excess of negative charge. Proof of the correctness of this assignment of charge has been found in the results of electrolysis of solutions of sodium copper biuret containing an excess of alkali to prevent hydrolysis. When a current is passed through

[4] The biuret molecule may exist in a number of tautomeric forms.
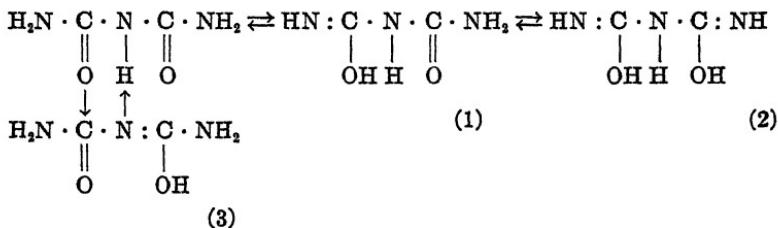
The formation of acid tautomers is assumed for other compounds of this type which show the biuret reaction.

the solution, the pink ingredient, which contains the copper, accumulates around the anode, and the solution surrounding the cathode becomes colorless.   Only after a relatively long time is metallic copper deposited upon the cathode showing the minimal ionization of the complex.

It may be recalled that the products of the reaction of acid imides with the biuret reagents have already been assigned the structures of salts comparable in constitution with the combinations of amino acids with salts; *e.g.*, $K^+(^-OOC \cdot CH_2 \cdot N^+H_3)Cl^-$.[1] For example, the product of the biuret reaction of the imide diethylbarbituric acid, $CO \cdot NH \cdot CO \cdot NH \cdot CO \cdot C\ (C_2H_5)_2$, is a substance of molecular formula $Na_2Cu(imide)_4 \cdot 2\ H_2O$, and may have, according to Rising and Johnson, the structure

$$Na_2^+ ([O^- \cdot C : N \cdot CO \cdot NH \cdot CO \cdot C(C_2H_5)_2]_2\ Cu^{++}\ (H_2O)_2)$$

$$[O^- \cdot C : N \cdot CO \cdot NH \cdot CO \cdot C(C_2H_5)_2]_2$$

in which the complex ion is assigned no excess of negative or positive charge.   The results of electrolysis of solutions of this salt indicate, however, that the complex ion containing copper is negative, since it moves to the anode.   In view of this fact it would be more accurate to assign to sodium copper diethylbarbitate the structure

$$Na_2^+ [(O^- \cdot C : N \cdot CO \cdot NH \cdot CO \cdot C(C_2H_5)_2)_4\ Cu^{++}\ (H_2O)_2]$$
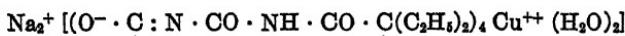
This structure indicates that sodium hydroxide neutralizes 2 molecules of diethylbarbituric acid; the imide then behaves as an amine, forming a complex with cupric ion in which there are 4 molecules of the imide.   Here, as in sodium copper biuret, six auxiliary valences of copper may function, two holding the oxygen atoms of 2 molecules of water, and each of the other four holding a nitrogen atom of the imide (or amide).

The pertinent question may well be raised at this point as to why 4 molecules of an acid imide react with the biuret reagents to form $Me_2Cu\ (imide)_4 \cdot xH_2O$, while di-acid amides[5] form with the

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[5] Biuret is an acid imide, with the characteristic group $-CO \cdot NH \cdot CO-$, as well as a di-acid amide.   In its reaction with alkali and cupric ion it behaves as a di-acid amide, since the product is a substance of formula Me_2Cu (di-acid amide)$_2$ rather than of formula $Me_2Cu(imide)_4$.

same reagents products of formula Me_2Cu (di-acid amide)$_2 \cdot xH_2O$. Some suggestions are offered in an attempt to formulate an answer to this question: an acid imide molecule contains 1 acid hydrogen atom, namely that in the group $- CO \cdot NH \cdot CO -$, capable of migration to a nearby oxygen atom under the influence of alkali, and of subsequent replacement by a metal ion. It may be reasoned that under the conditions used for the biuret reaction 2 molecules of imide, thus neutralized, and with amine properties thus enhanced could enter into a complex with cupric ion, forming the product

$$
Na_2^+ \left[\begin{array}{c} H_2C-C-O^- \\ | \quad \diagdown \\ | \quad\quad N \cdots\cdots Cu^{++} \cdots\cdots N \\ | \quad \diagup \\ H_2C-C=O \end{array} \qquad \begin{array}{c} ^-O-C-CH_2 \\ \diagup \quad | \\ \quad\quad | \\ \diagdown \quad | \\ O=C-CH_2 \end{array} \right]
$$
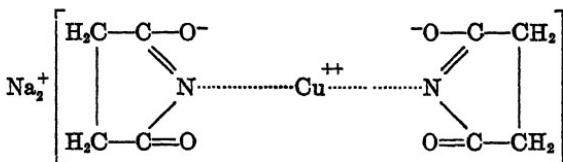
This product cannot, however, represent an actual molecule because it is not electrically neutral, since it carries an excess of two positive charges. Two further auxiliary valences of the copper may then function, resulting in the formation of an electrically neutral molecule, which contains 4 imide ions, of structure

$$
Na_2^+ \left[\begin{array}{cc} H_2C-C-O^- & ^-O-C-CH_2 \\ | \quad\quad N & N \quad\quad | \\ H_2C-C=O & O=C-CH_2 \\ & Cu^{++} \\ H_2C-C=O & O=C-CH_2 \\ | \quad\quad N & N \quad\quad | \\ H_2C-C-O^- & ^-O-C-CH_2 \end{array} \right]
$$
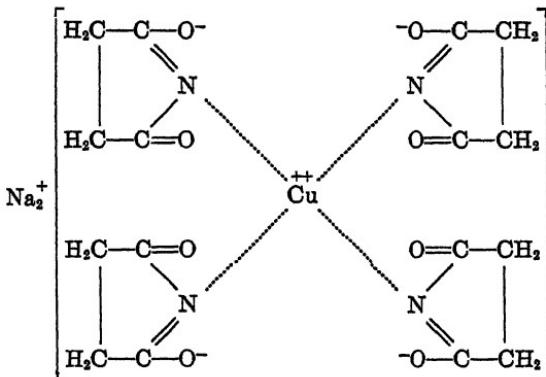
It is apparent that the complex now carries an excess of negative charge, as the results of electrolytic study indicate. In this molecule the second pair of imide ions fulfil the function of "internal neutralization," constituting the negative radical of the salt, much as $SO_4^=$ is the negative radical of $[Cu(NH_3)_4^{++}]$ $SO_4^=$, and

as Cl^- is the negative radical of $K^+(^-OOC \cdot CH_2N^+H_3)Cl^-$.   In the latter instances $SO_4^=$ and Cl^- are not placed within the complex since they are ionizable and not bound to any atom within the complex by auxiliary valences.   In the imide complex, however, the negative radical is placed within the complex since it is non-ionizable due to its secondary union with copper.   In summary, it requires 4 imide molecules to form an alkali copper imide salt since each molecule is a monobasic acid and contains 1 amine nitrogen atom.

If this summary represents the facts, and 4 hydrogen atoms and 4 nitrogen atoms are concerned in, and required for, formation of the product of a biuret reaction, it is readily seen that 2 molecules

TABLE II.
Analytical Data for Sodium Copper Malonamide.

Element.	Percentage composition.		Calculated percentage composition for $Na_2CuC_6H_{12}O_6N_4$ (mol. wt. 345.728).
	Sample I.	Sample II.	
Na	13.45	13.56	13.30
Cu	18.41	18.43	18.39
C	20.70	20.74	20.83
H	3.36	3.43	3.50
N	15.91	16.17	16.21
O			27.77
			100.00

of a di-acid amide fulfil these requirements, since a typical di-acid amide has in its molecule two acid groups and 2 amine nitrogen atoms.   The structure proposed for sodium copper biuret shows a molecule in which four auxiliary valences of copper find satisfaction in 2 molecules of the amide and two others in 2 molecules of water; an acid group in each molecule of biuret has been neutralized by 1 molecule of alkali, and the second acid group in each of the 2 biuret molecules is internally neutralized by the complex; these two acid groups thus fill the rôle of negative radical.

The biuret reaction of malonamide was next studied.   In a sense, malonamide is a more typical di-acid amide than is biuret since its molecule contains no acid imide group.   Sodium copper malonamide was obtained by treatment of 1 mol of malonamide with 4 mols of sodium hydroxide and 1 mol of cupric acetate in

water, and subsequent precipitation of the salt from the reaction mixture by means of alcohol and ether. The salt is a rose-pink amorphous powder which is decomposed by water and all acids. The analytical data for sodium copper malonamide are to be found in Table II.

The percentage composition of the salt agrees well with that calculated for a molecule of empirical formula $Na_2CuC_6H_{12}O_6N_4$, and this formula corresponds closely to Me_2Cu (di-acid amide)$_2 \cdot x$-H_2O (malonamide is $C_3H_6O_2N_2$, and $Na_2CuC_6H_{12}O_6N_4$ is Na_2Cu-$(C_3H_4O_2N_2)_2 \cdot 2H_2O$).[6] A structure for the salt is proposed.
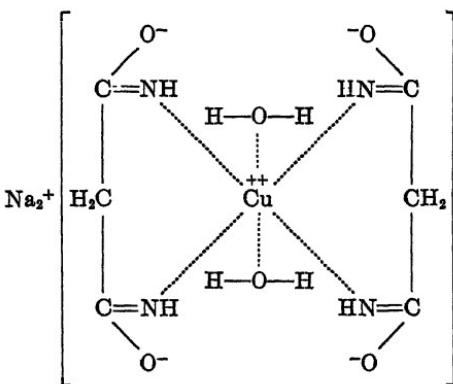
Sodium copper malonamide.

The close structural analogy of biuret and malonamide points to the probability that the alkali copper derivatives of the two di-acid amides are essentially the same in structure.

The effect of N-alkylation of di-acid amides upon the course of their biuret reaction was studied in the case of N-substituted oxamides and malonamides. Oxamide itself shows the biuret reaction very readily and the solid product of the reaction was isolated

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[6] Certain products of the biuret reaction of di-acid amides and acid imides (*e.g.*, sodium copper monoethyloxamide and potassium copper succinimide) are readily dehydrated *in vacuo* over sulfuric acid or phosphorus pentoxide at room temperature. It has seemed to us logical to assign to the water in such molecules a position outside the complex ion. Attempts to show the presence of water in sodium copper malonamide (and similarly in sodium copper biuret) by dehydration have failed. If there are 2 molecules of water in these salts they are probably within the complex.

by us but not in pure form, as the rose-colored salt was invariably contaminated with oxamide, the insolubility of which prevents its complete removal from the salt.   The analytical results for sodium copper oxamide so far available do not furnish any reliable information about the formula of the salt.

Monoethyloxamide, $C_2H_5NH \cdot CO \cdot CO \cdot NH_2$, was prepared and converted into its sodium copper derivative by treatment of 2 mols of monoethyloxamide with 2 mols of sodium hydroxide and 1 mol of cupric acetate in alcoholic solution containing approximately 15 per cent of water.   The salt was also prepared in water.   It is a rose-pink crystalline substance, separating as needles from alcohol, and plates from water.   The analytical data for the products of two preparations of the salt are to be found in Table III.

The percentage composition of the salt agrees well with that calculated for a molecule of empirical formula $Na_2CuC_8H_{20}O_8N_4$, and this formula corresponds closely to Me_2Cu (di-acid amide)$_2$-$\cdot xH_2O$ (monoethyloxamide is $C_4H_8O_2N_2$, and $Na_2CuC_8H_{20}O_8N_4$ is $Na_2Cu(C_4H_6O_2N_2)_2 \cdot 4H_2O$).   A structure for the salt is proposed.
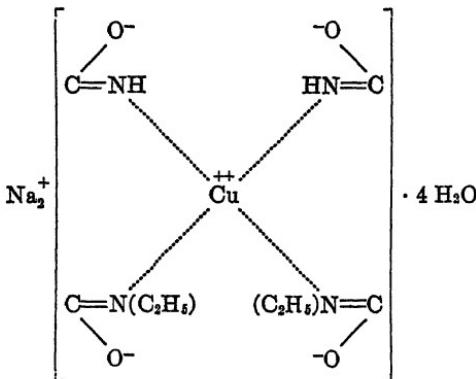
Sodium copper monoethyloxamide (rose, hydrated).

This structure finds support in (1) percentage composition data, (2) results of electrolysis of the salt, and (3) dehydration data.

Electrolysis.—The solution of the salt used for electrolysis contained an excess of alkali to prevent hydrolysis.   When a current was allowed to pass through this pink solution between suitable electrodes, the solution became colorless around the cathode within a short time and the line of demarcation between colored and color-

less regions of the solution moved gradually to the anode. In the pink solution about the anode there was formed slowly by the gradual decomposition of the complex a precipitate of copper oxide. It is to be concluded from these observations that copper is in a negative complex.

TABLE III.

Analytical Data for Sodium Copper Monoethyloxamide.

Element.	Percentage composition.		Calculated percentage composition for $Na_2CuC_8H_{20}O_8N_4$ (mol. wt. 409.73).
	Preparation I.	Preparation II.	
Na	11.19	11.36	11.23
Cu	15.62	15.43	15.52
C	23.53	23.96	23.43
H	5.01	4.96	4.92
N	13.21	13.06	13.67
O			31.23
			100.00

TABLE IV.

Analytical Data for Sodium Copper Diethyloxamide (Symmetrical).

Element.	Percentage composition.		Calculated percentage composition for $Na_2CuC_{12}H_{20}O_4N_4$ (mol. wt. 393.724).
	Sample I.	Sample II.	
Na	11.68	11.70	11.68
Cu	16.18	15.99	16.14
C	36.92	36.83	36.83
H	5.34	5.28	5.12
N	14.15	14.16	14.22
O			16.01
			100.00

Dehydration.—When the pink salt is allowed to stand in a desiccator over phosphorus pentoxide for a period of 13 days at room temperature it loses 17.04 per cent of its weight, an amount corresponding to 4 molecules of water. The loss in weight is accompanied by a change in color from pink to lavender, and this fact suggests that at least part of the water should be placed within the complex as water of structure. However, all 4 molecules are removable at low temperatures, resembling in this regard water of crystallization. We prefer to reserve judgment as to the struc-

tural position occupied by the water until further evidence is available.   The structure of the lavender salt may be
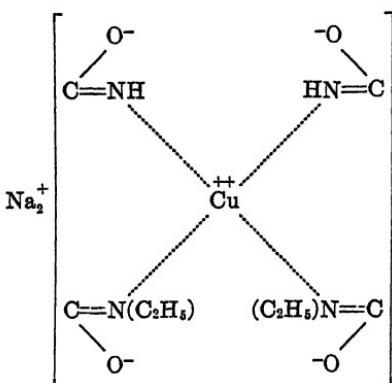
Sodium copper monoethyloxamide (lavender, dehydrated).

Besides the alkali copper derivatives of monoethyloxamide just described, sodium nickel monoethyloxamide was prepared and analyzed.   The analytical data for this salt are to be found in the experimental part of this paper.   It is a part of our plan to investigate alkali nickel salts of di-acid amides and imides.

Symmetrical diethyloxamide, $NH \cdot C_2H_5 \cdot CO \cdot CO \cdot NH \cdot C_2H_5$, was prepared and found to show the biuret reaction, although Schiff states that this di-acid amide does not react with the biuret reagents.[7]   A study of the conditions favorable for the occurrence of the reaction showed that diethyloxamide (symmetrical) forms no colored product with alkali and cupric ion in dilute aqueous solution.   When, however, precautions are taken to prevent hydrolysis by the use of a nearly non-aqueous reaction medium containing an excess of alkali and of diethyloxamide, the biuret reaction occurs, and a solid product of deep blue color is readily obtained.   Sodium copper diethyloxamide (symmetrical) was subjected to analysis, and the results are summarized in Table IV.

The percentage composition found agrees quite closely with that calculated for a molecule of empirical formula $Na_2CuC_{12}H_{20}O_4N_4$ (diethyloxamide is $C_6H_{12}O_2N_2$ and $Na_2CuC_{12}H_{20}O_4N_4$ is $Na_2Cu(C_6H_{12}O_2N_2)_2$).   Diethyloxamide (symmetrical) thus behaves as a typical di-acid amide in that it forms with alkali and cupric

[7] Schiff, H., *Ann. Chem.*, **299**, 257 (1898).

ion a product of formula Me_2Cu(di-acid amide)$_2$. The structure of the salt may be
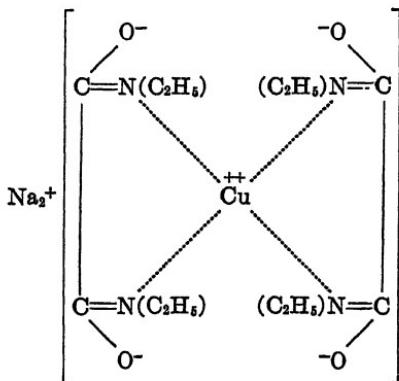
Sodium copper diethyloxamide (symmetrical).

Electrolysis showed that the copper is held in a negative complex.

The abrupt change in color from the rose of the sodium copper derivatives of oxamide and monoethyloxamide to the deep blue of sodium copper diethyloxamide (symmetrical) may perhaps be accounted for on the basis of a difference in water content. The pink biuret reaction products are considered to be hydrates. Dehydration of pink sodium copper monoethyloxamide produces a lavender salt. Blue sodium copper diethyloxamide (symmetrical) is not a hydrate. Possibly blue and lavender are the normal colors for anhydrous salts in the series.

Unsymmetrical diethyloxamide, $(C_2H_5)_2N \cdot CO \cdot CO \cdot NH_2$, and triethyloxamide, $(C_2H_5)_2N \cdot CO \cdot CO \cdot NH(C_2H_5)$, show no biuret reaction under any conditions so far used for the test. In view of these facts it seemed likely to us that tetraethyloxamide, $(C_2H_5)_2-N \cdot CO \cdot CO \cdot N(C_2H_5)_2$ would also be inactive towards the biuret reagents, and no attempt was made to devise a method for its synthesis.

The effect of N-substitution of alkyl groups in the malonamide series was next studied.[8] N-monoethylmalonamide, $C_2H_5 \cdot HN \cdot-$

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[8] It has been shown by Schiff (*Ann. Chem.*, **299**, 254 (1898)) that C-substituted di-acid amides of the type $NH_2 \cdot CO \cdot \overset{\overset{\textstyle H}{|}}{C} \cdot CO \cdot NH_2$ and $NH_2 \cdot-$ $\underset{\underset{\textstyle R}{|}}{}$

$CO \cdot CH_2 \cdot CO \cdot NH_2$, prepared by a method devised for the purpose, was found to react with cupric ion in alkali to form a bluish purple product.   So far the colored compound has not been isolated from the reaction medium in which it was formed, and there remains work to be done in this direction.   N-monophenylmalonamide, $C_6H_5 \cdot HN \cdot CO \cdot CH_2 \cdot CO \cdot NH_2$, said by Schiff not to show the biuret reaction, was found to form a greenish blue product with cupric ion in alkali.   The isolation of the pure compound has not yet been accomplished.   In this connection it is of interest to recall that copper glycine, $(NH_2 \cdot CH_2 \cdot CO_2)_2Cu$, is blue, while copper phenylglycine, $(C_6H_5 \cdot HN \cdot CH_2 \cdot CO_2)_2Cu$, is green.   N,N-diethylmalonamide, $C_2H_5 \cdot HN \cdot CH_2 \cdot CO \cdot NH \cdot C_2H_5$, was prepared and found to show no biuret reaction under the conditions used.

It is desired to state by way of summary certain hypotheses concerning the biuret reaction of di-acid amides, formulated by us on the basis of the results of the work just discussed.   Biuret, oxamide, and malonamide readily show the reaction; mono- and symmetrical dialkylated oxamides show the reaction but unsymmetrical dialkylated and trialkylated oxamides do not; N-monoalkylated malonamides show the reaction, while further alkylation of the malonamide molecule prevents it.   These findings are significant in that they serve to clarify the essential nature of the biuret reaction and aid in predicting with some degree of accuracy the behavior of di-acid amides toward the biuret reagents.

It has been pointed out previously[1] that a molecule which is sensitive to the biuret reaction is both an acid and a base and that the reaction of such a molecule with alkali and cupric ion involves salt, and complex ion, formation.   4 ionizable hydrogen atoms apparently take part in the reaction.   A typical acid imide mole-

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$$R$$
$$|$$
$CO \cdot C \cdot CO \cdot NH_2$ readily show the biuret reaction. Biltz, H., and
$$|$$
$$R$$

Jeltsch, A., *Ber. chem. Ges.*, **56**, 1923 (1923), have shown that di-acid
$$R$$
$$|$$
amides of the type $NH_2 \cdot CO \cdot N \cdot CO \cdot NH_2$ readily show the reaction. It is therefore apparent that substitution outside the amide groups does not affect the course of the reaction.

cule contains 1 such hydrogen atom, hence 4 imide molecules are to be found in the biuret reaction product of an acid imide, while a di-acid amide with 2 ionizable hydrogen atoms forms a product containing only 2 amide molecules. The bare essentials in the matter of atoms for the occurrence of the biuret reaction of a di-acid amide seem to us to be (1) 2 acid hydrogen atoms in each molecule for salt formation, and (2) 1 or more amine nitrogen atoms in each molecule for complex ion formation.

It would undoubtedly be apart from the truth, however, to conclude that the occurrence of these essential atoms in a di-acid amide molecule suffices to insure its reaction with cupric ion and alkali. Certain other conditions favoring the occurrence of the reaction must be met, and it is believed that a number of factors aside from the "essential atoms" mentioned influence the behavior of di-acid amides toward the biuret reagents. One of these may be termed the ionizing power of the amide as an acid. If the delicate balance between acidity and basicity in the amide molecule is disturbed, and, for example, the amide becomes so weakly basic (and therefore strongly acid) that complex ion formation is prevented, no biuret reaction can occur. On the other hand, if any change of condition makes the amide molecule so weakly acid (and consequently strongly basic) that salt formation is prevented, again no biuret reaction can occur. N-alkylation undoubtedly has a marked effect upon the acidity of the amide molecule. Since an alkylated amino group is more strongly basic than an unsubstituted one, an alkylated amide should be more strongly basic (and less acid) than its unsubstituted analogue. Mono- and symmetrical dialkylations of oxamide do not prevent the occurrence of a biuret reaction, and it is logical to conclude that such introduction of alkyl groups does not reduce the acidity of the molecule to the extent that salt formation cannot occur; 2 of the remaining hydrogen atoms in each molecule are evidently ionizable. Unsymmetrical diethyloxamide shows no biuret reaction despite the fact that its molecule contains 2 hydrogen atoms, and it would appear that substitution of this variety disturbs the balance between acidity and basicity to such an extent that the hydrogen atoms of the unsubstituted amino group are non-ionizable; the amide has no longer sufficient acid properties for salt formation, a fundamental preliminary to the occurrence of a biuret reaction. Triethyl-

oxamide is even more strongly basic and its 1 hydrogen atom may be wholly non-ionizable, while tetraethyloxamide has no acid properties whatever.

Thus in the oxamide series we have correlated the progressive decrease in acid strength attendant upon alkylation with decreased tendency to show the biuret reaction.

It has been of interest to us to note that the occurrence of the biuret reaction is prevented earlier in the biuret and malonamide, than in the oxamide, series.   It is reported by Biltz and Jeltsch[8] than monomethylbiuret, $CH_3 \cdot NH \cdot CO \cdot NH \cdot CO \cdot NH_2$, shows the reaction but that symmetrical dimethylbiuret, $CH_3 \cdot NH \cdot CO \cdot NH \cdot CO \cdot NH \cdot CH_3$, does not.   We have found that N-monoethylmalonamide shows the reaction, while symmetrical N-diethylmalonamide does not.   The inhibition of the reaction by the multiplication of alkyl groups attached to nitrogen may again be the result of the decrease in acid strength of the amide attendant upon alkylation. ·The cause of the earlier inhibition may be the separation of the two amide groups, in biuret by a nitrogen atom, and in malonamide by a carbon atom.   Just as oxalic acid, with its carboxyl groups adjacent, is a stronger acid than malonic acid with its carboxyl groups separated by a carbon atom (oxalic acid, $K_1 = 9 \times 10^{-2}$, $K_2 = 4 \times 10^{-5}$; malonic acid, $K_1 = 1.6 \times 10^{-3}$, $K_2 = 2 \times 10^{-6}$) so oxamide is probably a stronger acid than is malonamide or biuret, and for the same reason.   The influence of this "separation factor" upon di-acid amides may be such that the increase in the number of carbon or nitrogen atoms separating the amide groups weakens the strength of the amides as acids so that salt formation is ultimately hindered.   The failure of succinamide and of hydrazodicarbonamide to show the reaction may be explained on this basis (succinic acid, $K_1 = 7 \times 10^{-5}$, $K_2 = 2 \times 10^{-6}$, is even weaker than malonic acid).   Hence there are two factors which may affect the salt-forming power of di-acid amides and therefore their behavior with the biuret reagents, alkylation, and separation of the amide groups, and the net result of these influences may be no biuret reaction at all.

The effect of N-alkylation upon the acidity of the amide molecule has been stressed.   The effect of substitution upon basicity has also been observed in the case of phenylbiuret, $C_6H_5 \cdot NH \cdot CO \cdot$

$NH \cdot CO \cdot NH_2$, which does not show the biuret reaction.[9] The phenyl group is strongly acid in its effect and its substitution in the amino group may reduce the basicity of the group to such an extent that complex ion formation is prevented (aniline forms complexes less readily than does ammonia).

The conclusions outlined here are tentative, but are believed to be useful working tools for our further research upon the chemistry of the biuret reaction.

<div align="center">EXPERIMENTAL.</div>

1. Sodium Copper Biuret, $Na_2CuC_4H_{10}O_6N_6$.

The preparation of sodium copper biuret was carried out as follows: biuret[10] (4.0 gm.) was dissolved in a 10 per cent aqueous solution of sodium hydroxide (5.3 gm.) and powdered copper acetate $(Cu(C_2H_3O_2)_2 \cdot H_2O)$ (6.6 gm.)[11] was added at room temperature. The solution became deep violet-red in color. The contents of the reaction vessel were thoroughly stirred, and finally the supernatant solution was decanted from some unused copper acetate. To this solution there were added, with constant shaking, 20 volumes (600 cc.) of anhydrous alcohol containing a few cc. of sodium hydroxide solution, used to prevent hydrolysis of sodium copper biuret. Upon addition of the alcohol, sodium copper biuret was precipitated as a bright pink, finely divided substance. The salt was brought on a filter, washed with absolute alcohol, and dried in a vacuum desiccator over sulfuric acid and solid sodium hydroxide. From 4 gm. of biuret, 5.6 gm. of the sodium copper salt were obtained. It is particularly important that the alcohol and sodium hydroxide used in the preparation of all the biuret reaction products should be entirely free from carbon dioxide, and

[9] Gatewood, E., *J. Am. Chem. Soc.*, **45**, 146 (1923).

[10] Biuret was prepared by the method of Thiele (Thiele, J., *Ann. Chem.*, **303**, 95 (foot-note) (1898)) modified by Newburn, L., Master's thesis, University of Chicago (1923).

[11] The proportion of reagents used is 1 mol of biuret to 1 mol of copper acetate to 4 mols of alkali. The excess of alkali serves to prevent hydrolysis of the salt. The product of the reaction contains 2 molecules of biuret to every cupric ion, but it was found necessary to use an excess of copper salt in the preparation of all of our biuret reaction products, since products of varying copper content were obtained otherwise.

that all reagents used and products obtained be protected from moisture and carbon dioxide at all times.

Sodium copper biuret is a pink amorphous substance which is soluble in water, being extensively hydrolyzed therein. It is decomposed by carbon dioxide and all acids. When a solution of the salt was electrolyzed, the pink color deepened around the anode, while the solution around the cathode became colorless. It was concluded that the complex ion containing copper is negative.

A complete set of analytical data obtained for the salt (already referred to in the introduction to this paper) follows. The copper content of this salt and of the others about to be described was determined by electrolysis. Sodium was determined as sodium sulfate in solutions of the salts from which copper had been removed.

0.2269, 0.1927 gm. substance: 0.0918, 0.0793 gm. Na_2SO_4.
$Na_2CuC_4H_{10}O_6N_6$.   Calculated.   Na 13.23.
Found.   " 13.10, 13.32.
0.2269, 0.1927 gm. substance: 0.0416, 0.0353 gm. Cu.
$Na_2CuC_4H_{10}O_6N_6$.   Calculated.   Cu 18.28.
Found.   " 18.33, 18.32.
0.0719, 0.0848 gm. substance: 0.0362, 0.0429 gm. CO_2 and 0.0180, 0.0216 gm. H_2O.
$Na_2CuC_4H_{10}O_6N_6$.   Calculated.   C 13.81,   H 2.90.
Found.   " 13.73, 13.80,   " 2.80, 2.85.
0.0976, 0.1112 gm. substance: HCl (factor 0.0913) 18.33 cc., 21.11 cc.
$Na_2CuC_4H_{10}O_6N_6$.   Calculated.   N 24.17.
Found.   " 24.01, 24.27.

The analytical results agree well with the empirical formula $Na_2CuC_4H_{10}O_6N_6$. The structure which we have proposed for the salt is to be found in the introduction to this paper.

2. Sodium Copper Malonamide, $Na_2CuC_6H_{12}O_6N_4$.

To obtain sodium copper malonamide, malonamide[12] (3 gm.) was dissolved in a 10 per cent aqueous solution of sodium hydroxide (4.7 gm.), and this solution was treated with powdered copper acetate (5.9 gm.) at room temperature. After thorough mixing of these ingredients the reaction solution, which was deep purple,

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[12] Malonamide was prepared by the method of Freund, M., *Ber. chem. Ges.*, **17**, 133 (1884).

was decanted from the unused copper acetate and treated with 5 volumes of anhydrous alcohol, then very rapidly with 3 volumes of carbon dioxide-free anhydrous ether. Bright pink sodium copper malonamide was thereupon precipitated; the salt was collected on a filter, washed with absolute alcohol, and dried *in vacuo* over sulfuric acid and solid sodium hydroxide. From 3 gm. of malonamide 3.8 gm. of the salt were obtained.

Sodium copper malonamide is a pink amorphous substance which is soluble in water, in which it is appreciably hydrolyzed. In dilute solution the salt is decomposed and copper hydroxide is precipitated. This decomposition does not occur in a concentrated solution of the salt. Electrolysis of a 7 per cent solution of the salt led to the accumulation of the colored ingredient around the anode, while the solution around the cathode became colorless. The salt is decomposed by carbon dioxide and all acids. Attempts were made to prove that sodium copper malonamide is a hydrate. It was found, however, that the salt does not lose weight nor show a color change when placed *in vacuo* over boiling bromobenzene (156°). Above this temperature the compound turns brown and decomposes. Complete analytical data for the salt follows.

0.0869, 0.1232 gm. substance: 0.0361, 0.0516 gm. Na_2SO_4.
$Na_2CuC_6H_{12}O_6N_4$. Calculated. Na 13.30.
Found. " 13.45, 13.56.
0.0869, 0.1232 gm. substance: 0.0160, 0.0227 gm. Cu.
$Na_2CuC_6H_{12}O_6N_4$. Calculated. Cu 18.39.
Found. " 18.41, 18.43.
0.992, 0.0944 gm. substance: 0.0753, 0.0718 gm. CO_2 and 0.0298, 0.0289 gm. H_2O.
$Na_2CuC_6H_{12}O_6N_4$. Calculated. C 20.83, H 3.50.
Found. " 20.70, 20.74, " 3.36, 3.43.
0.3242, 0.1113 gm. substance: HCl (factor 0.0913) 40.35 cc., 14.08 cc.
$Na_2CuC_6H_{12}O_6N_4$. Calculated. N 16.21.
Found. " 15.91, 16.17.

The analytical results agree well with the empirical formula $Na_2CuC_6H_{12}O_6N_4$. The structure proposed for the salt is to be found in the introduction to this paper.

3. N-Monoethylmalonamide, $C_2H_5 \cdot HN \cdot CO \cdot CH_2 \cdot CO \cdot NH_2$, and Its Behavior with the Biuret Reagents.

N-Monoethylmalonamide was obtained from cyanoacetic ethyl ester in a series of reactions:

$C_2H_5O \cdot CO \cdot CH_2 \cdot CN + C_2H_5OH + HCl \rightarrow C_2H_5O \cdot CO \cdot CH \cdot C(OC_2H_5):$
NH_2Cl

$C_2H_5O \cdot CO \cdot CH_2 \cdot C(OC_2H_5) : NH_2Cl \overset{heat}{\rightarrow} C_2H_5O \cdot CO \cdot CH_2 \cdot CO \cdot NH_2$
$+ C_2H_5Cl$

$C_2H_5O \cdot CO \cdot CH_2 \cdot CO \cdot NH_2 + NH_2 \cdot C_2H_5 \rightarrow C_2H_5HN \cdot CO \cdot CH_2 \cdot CO \cdot NH_2$
$+ C_2H_5OH$
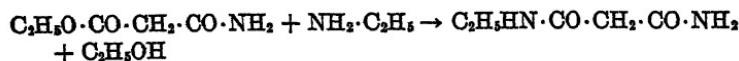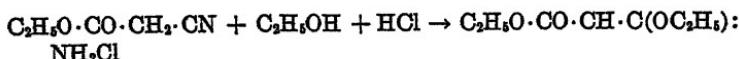
A. Malonamic Ethylimido Ester Hydrochloride, $C_2H_5O \cdot CO \cdot CH_2 \cdot$-
$C(OC_2H_5): NH_2Cl$.—The imido ester hydrochloride was prepared according to the method of Pinner,[13] a yield of 88.3 per cent being obtained.

B. Malonamic Ethyl Ester, $C_2H_5O \cdot CO \cdot CH_2 \cdot CO \cdot NH_2$.—The ester was obtained[14] in quantitative yield when the imido ester hydrochloride was warmed at a temperature of 106–108° till evolution of ethyl chloride ceased. The residue was extracted several times with anhydrous ether under a reflux; traces of ammonium chloride were separated from the ether solution by filtration, and the ether was removed by evaporation. The residue was pure amide ester of melting point 50°. The use of ether for extraction of the ester is much to be preferred to that of acetone advised by Pinner.

C. N-Ethylmalonamide, $C_2H_5HN \cdot CO \cdot CH_2 \cdot CO \cdot NH_2$.—Malonamic ethyl ester (14.5 gm.) in 10 cc. of 95 per cent alcohol was placed in an ice bath and ethylamine (10 gm.) was added; the containing flask was then stoppered and allowed to stand overnight. A solid mass of the ethyl amide formed, which was separated from the reaction solution and extracted, under a reflux, with 350 cc. of a mixture of equal amounts of dry benzene and toluene. The crystals of ethyl amide which formed upon cooling of the benzene-toluene mixture were separated by filtration and the mother liquor was used for several extractions of the crude mass of amide. The yield was 11.6 gm., or 77 per cent of the calculated yield. The ethyl amide melted at 122°. The substance was identified by analysis.

[13] Pinner, A., *Ber. chem. Ges.,* **28,** 479 (1895).
[14] It is probable that dehydration of the blue solution would result in the separation of sodium copper N-monoethylmalonamide in solid form. A method of dehydration has not yet been found. The reaction does not occur readily in other solvents.

0.1913, 0.1597 gm. substance: 0.3211, 0.2693 gm. CO_2 and 0.1374, 0.1159 gm. H_2O.

$C_5H_{10}O_2N_2$.   Calculated.   C 46.13,   H 7.75.
                     Found.   " 45.77, 45.99, " 8.03, 8.12.

0.1512, 0.1014 gm. substance: 29.5 cc. $N_2(23°, 747.0$ mm.), 19.8 cc. $N_2(24°, 741$ mm.) (over 50 per cent KOH).

$C_5H_{10}N_2O_2$.   Calculated.   N 21.52.
                     Found.   " 21.62, 21.33.   •

N-Monoethylmalonamide reacts with cupric ion and alkali. When 0.1 gm. of the amide is moistened with 1 drop of a saturated aqueous solution of copper acetate and 1 or 2 drops of 35 per cent aqueous sodium hydroxide a deep purplish blue color develops. Attempts to isolate this blue product have been so far unsuccessful and further study is needed.[14] Electrolysis of the blue solution led to a deepening of color around the anode, the solution around the cathode becoming colorless.

4. Sodium Copper Monoethyloxamide, $Na_2CuC_8H_{20}O_8N_4$.

To obtain sodium copper monoethyloxamide, monoethyloxamide[15] (3 gm.) was dissolved in 250 cc. of 95 per cent alcohol and this solution was warmed to 50°.   25 cc. of a warm aqueous solution containing 2.4 gm. of copper acetate were then added, whereupon a blue-green precipitate appeared.   A 35 per cent solution of sodium hydroxide (6 cc.) was dropped slowly into the reaction mixture from a burette while the mixture was constantly shaken. The blue-green precipitate was gradually replaced by a rose-pink one and precipitation was complete after $1\frac{1}{2}$ hours.   The precipitate was collected, washed with alcohol, and dried *in vacuo* over calcium chloride and soda-lime.   The yield was 5 gm.   The salt is a pink crystalline substance which is decomposed by water and all acids.   Electrolysis of a solution of the salt containing an excess of alkali to prevent hydrolysis resulted in intensification of the pink color around the anode while the solution around the cathode became colorless.   A precipitate of copper oxide formed under the anode as the colored ion containing copper decomposed, and copper was slowly deposited on the cathode.   Analytical data

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[15] Monoethyloxamide was prepared by the method of Wallach, O., *Ann. Chem.*, **184**, 65 (1877).

for the salt follow, analyses being carried out on the products of two different preparations of the salt.

0.6083, 0.7207 gm. substance: 0.2102, 0.2529 gm. Na_2SO_4.
$Na_2CuC_8H_{20}O_8N_4$.   Calculated.   Na 11.23.
                       Found.   "   11.19, 11.36.
0.6083, 0.7207 gm. substance: 0.0950, 0.1112 gm. Cu.
• $Na_2CuC_8H_{20}O_8N_4$.   Calculated.   Cu 15.52.
                       Found.   "   15.62, 15.43.

TABLE V.

Dehydration of Sodium Copper Monoethyloxamide.

Weight of samples.	Conditions used.	Time.	Loss in weight.		Color change.		
gm.		*hrs.*	*gm.*	*per cent*			
0.3504	P_2O_5, room temperature, *in vacuo*.	313	0.0597	17.04	Pink to lavender.		
0.3410	P_2O_5, over boiling alcohol, *in vacuo*.	168	0.0602	17.65	"	"	"
0.1082	Atmospheric pressure, 135°.	¾	0.0191	17.65	"	"	"
0.1003	" "	¾	0.0179	17.84	"	"	"

0.2283, 0.2552 gm. substance: 0.1970, 0.2242 gm. CO_2 and 0.1022, 0.1131 gm. H_2O.
$Na_2CuC_8H_{20}O_8N_4$.   Calculated.   C 23.43,   H 4.92.
                       Found.   "   23.53, 23.96, "   5.01, 4.96.
0.1743, 0.1322 gm. substance: 20.45 cc. N_2 (21°, 751.5 mm.) 15.25 cc. N_2 (20°, 752.4 mm.) (over 50 per cent KOH).
$Na_2CuC_8H_{20}O_8N_4$.   Calculated.   N   13.67.
                       Found.   "   13.21, 13.06.

The analytical results agree well with the empirical formula $Na_2CuC_8H_{20}O_8N_4$. The discussion of a possible structure for the salt is to be found in the introduction to this paper.

Dehydration Data.[16]—When the salt was obtained as just described, it was brought to constant weight *in vacuo* over calcium chloride and soda-lime to remove traces of water and alcohol.

It was then dehydrated (1) *in vacuo* over P_2O_5 and soda-lime at room temperature, (2) *in vacuo* over P_2O_5 and soda-lime over boil-

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[16] The dehydration data were obtained by DeKay and are stated in full in DeKay, H. G., Master's dissertation, University of Chicago (1930).

ing alcohol, (3) at atmospheric pressure at 135°.   The results are summarized in Table V.

It was impossible to remove the last traces of water at room temperature.   A further loss in weight occurred when the salt was warmed, ranging from 0.3 to 0.7 per cent.   If a molecule of molecular weight 409.73 were to lose 4 molecules of water the loss in weight would be 17.68 per cent.   Accordingly we have concluded that the salt contains 4 molecules of water.

5. Sodium Nickel Monoethyloxamide, $Na_3Ni_2C_{12}H_{37}N_6O_{14}$.

To obtain this salt a solution of monoethyloxamide (2 gm.) in 75 cc. of 95 per cent alcohol was warmed to 35–40° and 15 cc. of an aqueous solution containing 2 gm. of nickel acetate $(Ni(C_2H_3O_2)_2 \cdot 4\ H_2O)$ were added.   To this mixture 7.2 cc. of a 10 per cent aqueous solution of sodium hydroxide were added drop by drop from a burette with constant shaking of the reaction mixture.   A yellow precipitate formed, and precipitation was complete at the end of an hour.   It was observed that an appreciable amount of the amide remained unused.   The salt was collected on a filter, washed with absolute alcohol and dried *in vacuo* over calcium chloride.   A yield of 3 gm. was obtained.   The compound is yellow, crystalline, soluble in water, but quickly hydrolyzed and decomposed by it.   Upon warming, the salt is dehydrated, a loss in weight corresponding to 6 molecules of water occurring at 125°.   At 140° the loss in weight corresponds to 7 molecules of water.   At the latter temperature a color change from yellow to orange was observed.

The analytical data for the salt follows.   Sodium was determined as sodium sulfate in the filtrate from precipitation of nickel as nickel dimethylglyoxime.   The nickel was determined as nickel dimethylglyoxime.

0.2749, 0.2705 gm. substance: 0.0799, 0.0781 gm. Na_2SO_4.

$Na_3Ni_2C_{12}H_{37}N_6O_{14}$.   Calculated.   Na 10.21.
Found.   "   9.41. 9.35.

0.2749, 0.2705 gm. substance: 0.2365, 0.2349 gm. Ni.

$Na_3Ni_2C_{12}H_{37}N_6O_{14}$.   Calculated.   Ni 17.37.
Found.   "   17.48, 17.65.

0.2108, 0.2167 gm. substance: 0.1651, 0.1714 gm. CO_2 and 0.1082, 0.1104 gm. H_2O.

$Na_3Ni_2C_{12}H_{37}N_6O_{14}$.   Calculated.   C 21.32,   H 5.52.

Found.   " 21.36, 21.57, " 5.59, 5.71.

0.1253, 0.1071 gm. substance: 13.7 cc. $N_2(22°,$ 751.0 mm.), 11.9 cc. N_2 (24.5°, 748.5 mm.) (over 50 per cent KOH).

$Na_3Ni_2C_{12}H_{37}N_6O_{14}$.   Calculated.   N 12.43.

Found.   " 12.26, 12.26.

The analytical results agree well with the empirical formula $Na_3Ni_2C_{12}H_{37}N_6O_{14}$. Evidence upon which to base a structure for the salt is not available; its molecule is obviously more complex than are those of the sodium copper salts studied. The salt is a hydrate, as already stated. The nickel is bivalent, since hydrolysis and electrolysis of the salt produce nickelous hydroxide. In the electrolysis the yellow ingredient of the solution accumulates a-round the anode and nickel hydroxide is precipitated, hence the complex ion contains nickel and is negative.

6. Sodium Copper (Symmetrical) Diethyloxamide, $Na_2CuC_{12}H_{20}N_4O_4$.

The salt was prepared as follows: A solution of copper acetate (2 gm.) in 200 cc. of 95 per cent alcohol was warmed to 60° and filtered into a solution of 4 gm. of symmetrical diethyloxamide[17] in 100 cc. of 95 per cent alcohol, also at 60°. The mixture was warmed almost to boiling, 8 cc. of 35 per cent aqueous sodium hydroxide solution being meanwhile dropped slowly into it. The dark blue reaction mixture was filtered from any residue without washing of the residue, since treatment of the filtrate with alcohol or ether decomposed the sodium copper salt in it. A heavy mass of dark blue crystals formed in the filtrate, and these were collected and dried in a vacuum desiccator. The yield of salt was 3.25 gm.

Sodium copper (symmetrical) diethyloxamide is a dark blue crystalline substance which is extremely hygroscopic. It is readily hydrolyzed. Electrolysis of an aqueous solution made stable by the addition of a large excess of alkali gave the usual results: the copper was found in the blue complex at the anode, while the solution around the cathode became colorless.

Analyses of the salt were carried out as usual except that after

[17] Diethyloxamide (symmetrical) was prepared according to the method of Wallach, O., *Ann. Chem.*, **184**, 33 (1877); **214**, 268 (1882).

the determination of copper by electrolysis the organic matter was destroyed by treatment with hot concentrated H_2SO_4 and 30 per cent H_2O_2 before sodium was determined as sulfate.

0.6048, 0.6217 gm. substance: 0.2185, 0.2263 gm. Na_2SO_4.
$Na_2CuC_{12}H_{20}N_4O_4$. Calculated. Na 11.68.
Found.   "   11.70,   11.78.
0.6048, 0.2401 gm. substance: 0.0959, 0.0384 gm. Cu.
$Na_2CuC_{12}H_{20}N_4O_4$. Calculated. Cu 16.14.
Found.   "   15.87,   15.99.
0.2216, 0.2493 gm. substance: 0.2993, 0.3346 gm. CO_2 and 0.1075, 0.1176 gm. H_2O.
$Na_2CuC_{12}H_{20}N_4O_4$. Calculated. C 36.83,   H 5.12.
Found.   " 36.83, 36.60, " 5.43, 5.28.
0.1133, 0.1132 gm. substance: 14.00 cc. N_2 (19°, 758.5 mm.) and 14.15 cc. N_2 (22°, 753.5 mm.) (over 50 per cent KOH).
$Na_2CuC_{12}H_{20}N_4O_4$. Calculated. N 14.22.
Found.   " 14.16, 14.04.

The hydrogen content is slightly high for a molecule of empirical formula $Na_2CuC_{12}H_{20}N_4O_4$, due possibly to the extremely hygroscopic nature of the compound. Otherwise the analytical results agree well with this empirical formula.

<center>SUMMARY.</center>

1. Further work upon the chemistry of the biuret reaction is reported.

2. The present paper discusses the biuret reaction of the typical di-acid amides biuret, malonamide, and oxamide. The sodium copper salts of the first two of these were isolated and analyzed, empirical formulas for them deduced, and structures proposed.

3. This paper discusses also the biuret reaction of the substituted di-acid amides N-monoethylmalonamide, monoethyloxamide, and symmetrical diethyloxamide. Their sodium copper salts were isolated and analyzed, empirical formulas deduced, and structures proposed.

4. The theory of the biuret reaction is augmented with regard to the atoms concerned in the biuret reaction of di-acid amides.

5. An explanation is offered for the inhibition of the biuret reaction of di-acid amides by multiple substitution of alkyl groups in their molecules, and by separation of the amide groups by carbon or nitrogen atoms.

ARSENIC DERIVATIVES OF CYSTEINE.

By J. M. JOHNSON AND CARL VOEGTLIN.

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In previous papers from this laboratory (1–3) it was shown that reduced glutathione (crystalline) and certain other sulfhydryl compounds protect rats and trypanosomes against the toxic action of arsenious acid and its aromatic derivatives ($R \cdot AsO$). It was also shown that As_2O_3 and 3-amino-4-hydroxyphenylarsenious oxide inhibit the rate of oxidation of SH glutathione by molecular oxygen in the presence of traces of hemin as catalyst. The action of these sulfhydryl compounds is quite specific and depends on the presence of the SH group. Amino acids (except cysteine), glucose, and other tissue constituents are inactive. The theory was therefore advanced that the protective action of SH compounds is due to their chemical affinity for arsenious oxides

$$R \cdot AsO + \begin{matrix} HS \cdot R \\ HS \cdot R \end{matrix} = R \cdot As \begin{matrix} S \cdot R \\ S \cdot R \end{matrix}$$
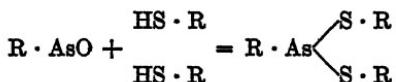
and the biological action of arsenic was explained by the assumption that arsenic combines chemically with cellular SH compounds which are essential to the life of the cell. In order to add further evidence the following arsenic derivatives of cysteine were prepared.

3-Amino-4-hydroxyphenylarsenious oxide, prepared by the method of Ehrlich and Bertheim (4), in the form of its hydrochloride, may be made to react in alcoholic solution with cysteine hydrochloride to form a crystalline compound. The compound obtained thus is the hydrochloride, but after carrying out this reaction a great many times we could not obtain a compound containing a definite amount of hydrochloric acid. But we found

that by neutralizing the aqueous solution of the reaction product with sodium hydroxide and acidifying with acetic acid a crystalline compound can be obtained, which is free from hydrochloric acid.   The reaction proceeds as follows:

$$\underset{\underset{\overset{|}{OH}}{\underset{NH_2HCl}{\bigcirc}}}{AsO} + 2\ \underset{\underset{COOH}{\overset{|}{CH \cdot NH_2HCl}}}{\overset{CH_2SH}{\overset{|}{\ }}} = \underset{\underset{\overset{|}{OH}}{\underset{NH_2HCl}{\bigcirc}}}{\overset{\overset{SCH_2CHNH_2HClCOOH}{\diagup}}{As—SCH_2CHNH_2HClCOOH}} + H_2O$$
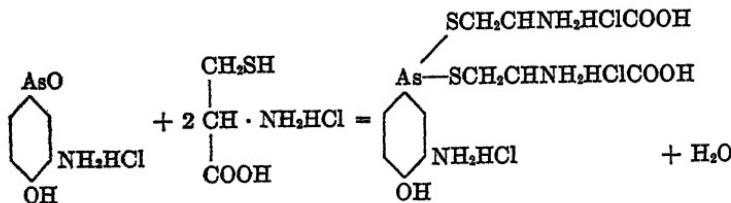
The hydrochloric acid so combined was then removed by the sodium hydroxide-acetic acid treatment and the compound finally formed in beautiful crystalline condition is:

$$\underset{\underset{\overset{|}{OH}}{\underset{NH_2}{\bigcirc}}}{As\underset{\diagdown SCH_2CH\ NH_2\ COOH}{\overset{\diagup SCH_2CH\ NH_2\ COOH}{\big\langle}}}$$
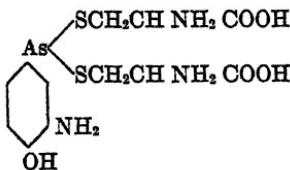
In carrying out the reaction we used an excess of cysteine hydrochloride so that there could be no question of merely a mechanical mixture having come out.   Furthermore the solubilities of the compound formed are entirely different from those of the substances from which it is made.

Preparation.—6.30 gm. (3 mols) of pure cysteine hydrochloride were dissolved in 200 cc. of absolute ethyl alcohol.   The solution was filtered from a slight amount of insoluble material (perhaps cystine).   To the above solution there was added a solution of 3.52 gm. (1 mol) of 3-amino-4-hydroxyphenylarsenious oxide hydrochloride dissolved in 50 cc. of absolute ethyl alcohol.   The mixture was then placed on the water bath and heated under a reflux condenser while a stream of dry hydrogen gas was passed through the solution.   The heating was continued for 3 hours. During this time there was formed an insoluble compound, slightly pink in color.   The mixture was now cooled to room temperature and the solid separated by centrifugation.   It was then washed three times with 75 cc. of absolute ethyl alcohol.   This was done

also by centrifugation. The solid substance was now dissolved in 100 cc. of distilled water; the solution obtained was neutralized with dilute sodium hydroxide and then acidified with acetic acid. In a short time the solution began to fill with crystals. On standing overnight it had set to a mass of almost white crystals, seen to be small needles under the microscope. The crystals were filtered off and washed three times with water. They were now recrystallized by dissolving in hot water heated up to the boiling point, filtering, and cooling. 1.9 gm. of substance were obtained after filtering and drying to constant weight in a vacuum desiccator over P_2O_5.

Analysis.
Calculated for $C_{12}H_{18}O_5N_3S_2As$.   S 15.15, As 17.72.
Found.   S 15.08, 15.00, 15.17; As 18.06, 17.77, 17.94.

A melting point determination showed decomposition at 225–227° (uncorrected).

Two samples of the substance dissolved in 2 per cent hydrochloric acid and titrated with 0.01 N iodine in presence of potassium iodide consumed 9.34 and 9.38 cc. respectively for 10 mg. of substance. By calculation the arsenic and sulfur should consume 4.72 and 4.73 cc. respectively or a total of 9.45 cc. of 0.01 N iodine.

Cysteine hydrochloride in alcoholic solution may be made to react with arsenious chloride, also in alcoholic solution. There is formed a compound which does not give a constant analysis for hydrochloric acid. However, by neutralization with sodium hydroxide and acidification with acetic acid a beautifully crystalline compound may be obtained which can be recrystallized from hot water. The reaction proceeds as follows:

$$AsCl_3 + 3 \begin{array}{c} CH_2\,SH \\ | \\ CH\!-\!NH_2\,HCl \\ | \\ COOH \end{array} = As \Big\langle \begin{array}{l} SCH_2\,CHNH_2HClCOOH \\ SCH_2\,CHNH_2HClCOOH \\ S\,CH_2CHNH_2HClCOOH \end{array} + 3\,HCl$$
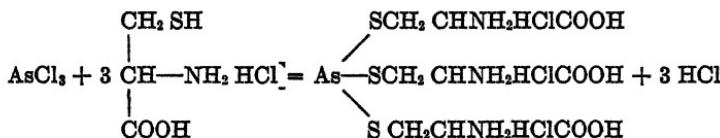
Preparation.—1.64 gm. of $AsCl_3$ (1 mol) were dissolved in 10 cc. of absolute alcohol and mixed with a solution of 5.73 gm. (4 mols) of cysteine hydrochloride in 250 cc. of absolute alcohol. The mixture was allowed to stand at room temperature. In a short while a white

precipitate began to come out.   In order to complete the precipita-
tion, it was allowed to stand at room temperature overnight.   The
white compound was then centrifuged off and washed several
times with absolute alcohol.   The substance was now dissolved
in 100 cc. of water with the aid of sodium hydroxide.   It was then
made slightly acid with acetic acid.   The white precipitate formed
was centrifuged off and washed repeatedly with water until free
from chlorides.   It was now dissolved in hot water, filtered, and
allowed to stand at room temperature.   It formed a mass of beau-
tiful white felted needles.   These were filtered off and dried in a
vacuum desiccator.   Yield 2.41 gm.   A melting point determina-
tion showed decomposition at 260° (uncorrected).

Two samples of the substance were dissolved in 2 per cent hy-
drochloric acid and titrated with 0.01 N iodine after the addition of
potassium iodide.   They used up 6.56 and 6.64 cc. respectively, of
iodine per 10 mg. of substance.   The sulfur alone in the substance
should use up by calculation 6.89 cc. of 0.01 N iodine.

Analysis.
Calculated for $C_9H_{16}O_6N_3S_3As$.   S 22.10, As 17.22.
Found.   S 22.39, 22.26, 22.42; As 17.24, 17.09, 17.58.

The ease with which arsenious oxides react with cysteine with
the formation of pure crystalline compounds adds further chemical
evidence to our theory of the biological action of arsenic.   The
chemotherapeutic action of these arsenic compounds will be in-
vestigated.

The fact that in the iodometric titration of the compound made
from 3-amino-4-hydroxyphenylarsenious oxide and cysteine an
amount of iodine is used up which corresponds to the sum required
for the oxidation of the sulfur and arsenic calls for comment.
Brown and Kolmer (5) have attempted to estimate the SH gluta-
thione of the tissues of normal animals and of animals having re-
ceived arsenic treatment by means of iodine titration of protein-
free extracts.   It is obvious that this method could not possibly
yield results which could be easily interpreted, as indicating com-
bination or absence of combination of administered arsenic with
tissue SH compounds.

Since this manuscript was written, a paper by Labes (6) has
come to our attention, in which there is described a crystalline

compound made from cysteine and As_2O_3. This preparation must have been impure, as analysis showed that for each atom of arsenic there were 3.22 atoms of sulfur.

SUMMARY.

The synthesis of tricysteinylarsine and dicysteinyl-3-amino-4-hydroxyphenylarsine is described. ` Both substances were obtained in pure crystalline form.

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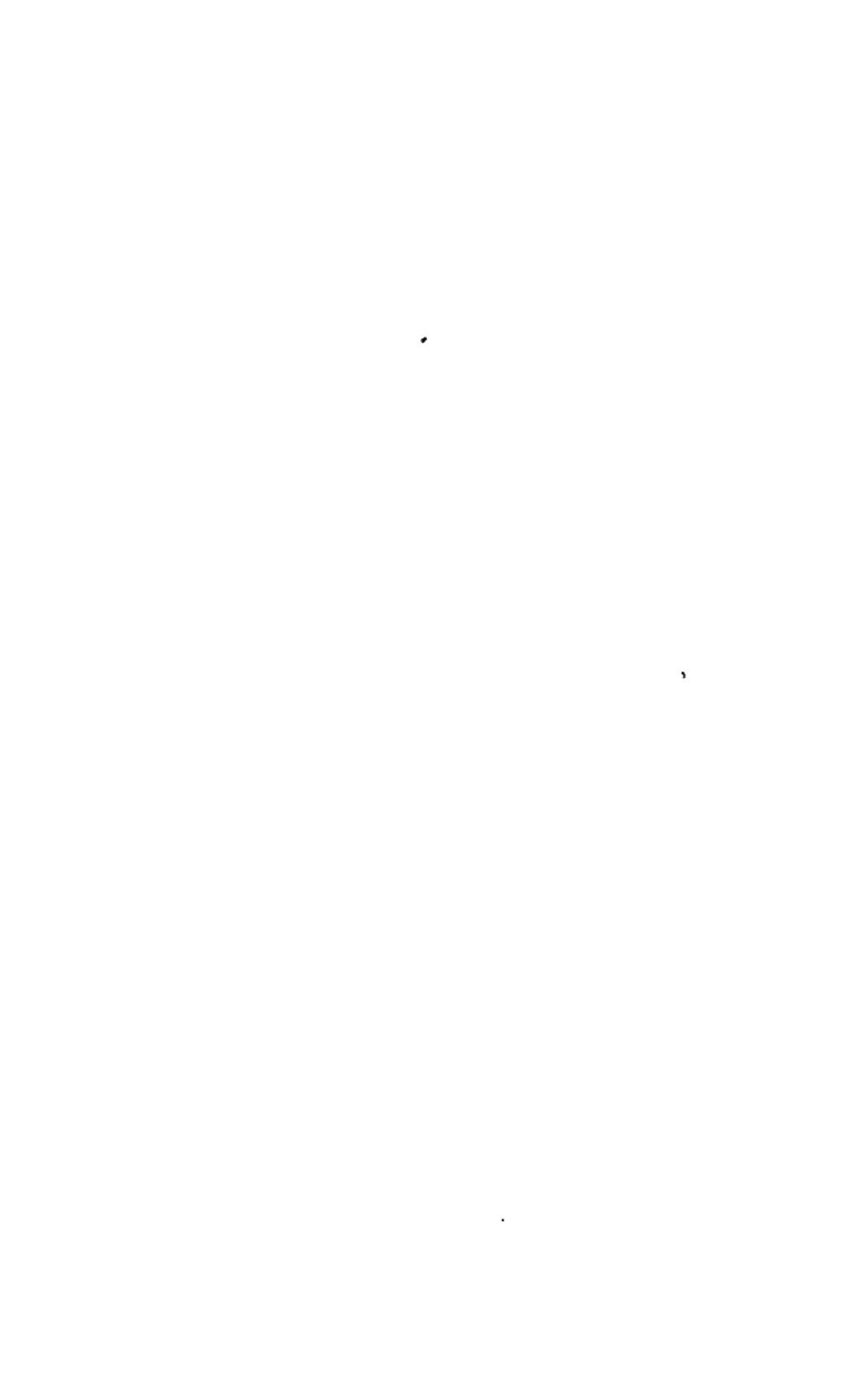
ON RABBIT LIVER GLYCOGEN AND ITS PREPARATION.

By MELVILLE SAHYUN and CARL L. ALSBERG.

(From the Department of Chemistry and the Food Research Institute, Stanford University, California.)

The importance of studying glycogen that has been prepared without the use of strong caustic alkali has been pointed out in previous papers.[1,2] In one of them (Petree and Alsberg), a method of preparation based on the removal of protein with picric acid was described. In the present paper, an improved method with trichloroacetic acid is presented, and rabbit liver glycogen prepared by it is described with especial reference to its phosphorus content.

Trichloroacetic Acid Method for Preparation of Glycogen.

The rabbit livers were rapidly removed from the animals, cut to pieces, and thoroughly ground in a mortar with 3 parts of 3 per cent trichloroacetic acid solution. The ground mixture was then strained through cheese-cloth and pressed by hand. The pressed tissues were replaced in the mortar and thoroughly mixed with fresh 3 per cent trichloroacetic acid solution. This second extraction was found essential, for about 30 per cent, roughly, of the glycogen remains in the ground, pressed tissues. A third extraction might also be worth while.[3] The various extracts were collected and centrifuged, since the rate of filtration was very slow. To 1 volume of the opalescent filtrate, 2 volumes of 95 per cent ethyl alcohol were added. The glycogen precipitated

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[1] McDowell, M., *Proc. Soc. Exp. Biol. and Med.*, **25**, 85 (1927).

[2] Petree, L. G., and Alsberg, C. L., *J. Biol. Chem.*, **82**, 385 (1929).

[3] In this regard, we might mention an experiment with the tissues after the second extraction. About one-third of the tissue residue was kept in trichloroacetic acid for about 1 week. 0.7 gm. of glycogen was recovered.

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rapidly, and upon standing for a short time it settled at the bottom of the vessel, so that most of the liquid could be decanted. The glycogen was then centrifuged and the remaining liquid decanted. It was then redissolved in a small amount of 3 per cent trichloroacetic acid and centrifuged to remove any porteins still present. The trichloroacetic acid-glycogen solution was then shaken with ether several times in a separatory funnel to remove any fatty acids present. The extracted solution was then precipitated with 2 volumes of alcohol and centrifuged. The centrifuged glycogen was redissolved in distilled water, precipitated with alcohol, then centrifuged, and the process repeated twice. If the glycogen solution is so dilute that it does not precipitate rapidly upon the addition of alcohol, a trace of solid ammonium acetate added to the mixture will hasten precipitation. Ammonium acetate is superior to either sodium or potassium salts, since it does not appear in the ash and is soluble in dilute alcohol. From the dilute alcohols decanted in the course of preparation, an appreciable amount of glycogen can be recovered by allowing them to stand for several days.

The glycogen is finally taken up in the least possible amount of water and filtered through a Büchner filter, precipitated with alcohol, taken up in 95 per cent alcohol, and finally in absolute alcohol before drying. Ether was not used, for it dries the surface too rapidly and leaves the interior wet. The glycogen was dried *in vacuo* over calcium chloride. In this way, Preparation A was produced. From 240 gm. of liver used for one of the preparations, a total of 18 gm. of glycogen in the dry form was obtained.

Drying to constant weight in the above manner required a minimum of 2 weeks at room temperature with frequent stirring. It is not safe to dry at elevated temperatures, as the following experience demonstrates.

While drying about 40 gm. of glycogen, prepared during the summer of 1929, in an oven at 60°, the temperature rose accidentally to 105° and caused alteration in the nature of the preparation. Since it acquired different physical and, perhaps, chemical properties, we speak of it as "denatured" glycogen. Its color changed from pure white to slightly brown. The viscosity of its solutions was greatly increased, though no measurements were made. It

became less soluble, and in strong solutions most of it settled at the bottom of the beaker in the form of a sticky, gelatinous mass. The weak solutions of glycogen above the gelatinous mass and this gelatinous material, itself, had the same characteristics. It was filtered through charcoal to remove impurities, and then subjected to boiling with 60 per cent potassium hydroxide solution for more than 7 hours under a reflux condenser in order to destroy other substances than glycogen that might have been formed. The process of purification by precipitation with alcohol was then repeated. Upon drying the final product a shining, transparent surface of extreme hardness was formed on the outside that retarded drying to a considerable extent, and we, therefore, believe that about 10 per cent by weight of water remained present. This "denatured" glycogen is designated as Preparation B.

The removal of proteins by means of the Folin-Wu sodium tungstate and sulfuric acid reagent was also investigated. The substitution of these reagents for trichloroacetic acid is not advantageous, since tungstic acid is precipitated by alcohol along with glycogen. However, investigations are in progress to determine the value of these reagents for a quantitative, rapid, micro estimation of glycogen in tissues.

Analysis of Glycogen.

Ash.—The ash content of glycogen preparations depends to a large extent upon (1) the method of preparation, (2) the source, and (3) the presence of such ions as calcium, sodium, potassium, iron, etc.

McDowell,[1] using Pflüger's method, reported the ash content of a glycogen preparation from the edible mussel, *Mytilus edulis*, as 0.254 per cent before electrodialysis with ultrafiltration, and 0.0487 to 0.0828 per cent after dialysis. Petree and Alsberg,[2] using the picric acid method, obtained from abalone, *Haliotis rufescens*, Swainson, preparations with 0.069 per cent before dialysis, and 0.057 per cent after dialysis. In a more recent investigation of glycogen, Barbour[4] prepared his samples from livers of rabbits by Pflüger's method, and reported·that his preparation was 99 to 100 per cent pure. He did not state the ash

[4] Barbour, A. D., *J. Biol. Chem.*, **85**, 29 (1929).

content exactly. Though the ash content of glycogen, carefully prepared, is in general very small, yet it cannot be regarded as negligible. Earlier investigations on this subject are reviewed by Petree and Alsberg.[2]

Preparation A was dried to constant weight in a finely pulverized form, and the ash content of each of two samples of 500 mg. determined. Results were as follows:

Weight of ash.	Per cent ash.	Per cent P_2O_5.	Ca	Fe
gm.				
0.0009	0.18	16.5	Trace.	Trace.
0.0010	0.20	15.5	"	"

It is quite possible to obtain ash-free glycogen, in spite of the fact that phosphorus may be present, for unless there are such ions as calcium, iron, sodium, potassium, etc., phosphorus will volatilize upon ashing. In the presence of such ions, however, phosphate is formed, and consequently they determine the percentage of ash formed.

Reducing Sugars.—Though Preparation A had all the appearance of a pure material, its purity was verified by hydrolyzing a portion of it in order to determine the reducing sugar equivalent as glucose, and from the value obtained the amount of glycogen was computed by multiplying by the factor 0.927.

Two samples of Preparation A and two others of Preparation B were accurately weighed and dissolved in 100 cc. of distilled water in a 250 cc. volumetric flask. The weight of each sample was 1 gm. 2.2 per cent of hydrochloric acid was added to each solution, and the flasks were placed in boiling water for 3 hours. Upon cooling, the samples were neutralized to litmus with sodium hydroxide and made up to volume. The results for reducing sugars, determined by the sugar method of Folin and Wu, and recorded as glucose, were as follows:

Preparation.	Weight.	Sugars as glucose.	Glycogen computed.
	gm.	gm.	gm.
A	1.000	1.040	0.964
	1.000	1.075	0.996
B	1.000	0.980	0.908
	1.000	1.010	0.936

As can be seen from the data, the glycogen value approaches very closely the theoretical value for Preparation A, but is too low for Preparation B, which is to be expected since this preparation is "denatured" and not anhydrous.

Phosphorus.—While there is as yet no final and absolute proof that phosphorus as P_2O_5 is an integral component of the glycogen molecule, the investigations of McDowell[1] and Petree and Alsberg[2] support that view, since they were unable to free glycogen from phosphorus by means of prolonged electrodialysis with ultrafiltration. The amounts reported are small. The literature has been reviewed by Petree and Alsberg.[2] In a recent investigation by McBride[5] on glycogen, phosphorus was determined and found to agree with the findings of former investigators.

When a sample of Preparation A was dissolved in water, it showed no positive test for phosphorus. Preparation B, "denatured" glycogen, also gave negative results when freshly dissolved, but upon standing 24 hours the same solution gave a positive test.

After hydrolysis, however, tests for phosphoric acid were positive. Hydrolysis was accomplished with hydrochloric acid, as described above in connection with the estimation of reducing sugar. Two samples of Preparation A and two of Preparation B were hydrolyzed and phosphoric acid estimated by the method of Fiske and Subbarow.[6] The reagents used were thoroughly tested and found free from phosphorus. The method is quite satisfactory, as the close checks obtained in analyzing a sample of commercial takadiastase indicated.

100 mg. of takadiastase were dissolved in a 100 cc. volumetric flask, and samples were removed and analyzed for phosphorus. 50 cc. of this solution were electrodialyzed and the ultrafiltrable fraction as well as the non-ultrafiltrable fraction were analyzed.

Taka-diastase.	Total P as P_2O_5	Ultra-filtrable P_2O_5.	Non-ultra-filtrable P_2O_5	Total ultra-filtrable and non-ultrafiltrable P_2O_5.
mg.	mg.	mg.	mg.	mg.
100	2.04	1.85	0.215	2.06
	2.05	1.82	0.210	2.03
100	2.04	1.83	0.210	2.04
		1.82		

[5] McBride, J. J., A chemical study of glycogen and its preparation, Dissertation, Columbia University (1929).

[6] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 66, 375 (1925).

Two samples of glycogen, Preparation A, and two others of "denatured" glycogen, Preparation B, were hydrolyzed with hydrochloric acid, as described above. The results of the phosphorus determinations were as follows:

Preparation.	Weight of glycogen hydrolyzed.	Per cent of P as P₂O₅.
	mg.	
A	1000	0.031
	1000	0.033
B	1000	0.028
	1000	0.029

Though we employed a different procedure, a different source of glycogen, and a different method of phosphorus determination from McDowell,[1] Petree and Alsberg,[2] and McBride,[5] yet our results are in general agreement with their findings.

Attention is to be called to the fact that in Preparation B the glycogen had been subjected during preparation to drastic treatments such as filtering through charcoal and boiling for 7 hours in a solution of 60 per cent potassium hydroxide; yet the phosphorus content was only slightly less than that of Preparation A, although Preparation B was not anhydrous and distinctly altered.

Aside from its relative insolubility, noted above, the characteristics of Preparation B, so far as investigated, were as follows:

Iodine Test.—A solution gave the same port wine color with iodine as ordinary glycogen, but it differed from the pure preparation in showing a change of color in fading. Instead of the original color's becoming gradually fainter, the color turned pink as it vanished.

Opalescence.—It was considerably less opalescent, so that 4 to 5 times the concentration was required to match the opalescence of pure glycogen.

Reducing Sugars.—It did not reduce Benedict's alkaline copper solution and did not precipitate silver from an ammoniacal silver nitrate solution.

Adhesion.—It acquired very strong adhesive properties, even to smooth surfaces, such as glass.

SUMMARY.

A method is described for the preparation of glycogen with the use of 3 per cent trichloroacetic acid.

Glycogen prepared by this method was found to contain between 0.20 and 0.18 per cent ash. The samples analyzed were not dialyzed. The ash was found to contain phosphorus as P_2O_5 and traces of calcium and iron.

Upon hydrolysis, the computed value of this glycogen was from 0.964 to 0.996 per cent of the theoretical.

The phosphorus content, calculated as P_2O_5, of this glycogen was 0.032 per cent.

Glycogen is very easily altered by heating to 105°. Some of the properties of such a glycogen are described.

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A NEW SYNTHESIS OF ASPARTIC ACID.*

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During the past 80 years the following methods for synthesizing aspartic ,acid have been reported; the decomposition of acid ammonium malate by heat (1), the racemization of active aspartic acid (2) and active asparagine (3), the reaction of maleic and fumaric acids with ammonia in a closed tube (4), the reduction of oxalacetic ester oxime (5), the reduction of silver fumarate by hydroxylamine hydrochloride (6), the reduction of nitrosuccinic ester (7), the catalytic reduction and aminization of oxalacetic acid (8), and the hydrolysis of ethane-α-amino-α,α,β-tricarboxylic acid triethyl ester (9).

None of these procedures appears to be entirely satisfactory. The direct aminization of fumaric or maleic acid should give good results although it has the disadvantage of requiring high pressure operation. The recent method of Keimatsu and Kato (9) has been carefully worked out but the use of sensitive intermediate substances and tedious manipulations are involved while a considerable quantity of iminodiacetic acid is formed in addition to the desired substance.

The accompanying reactions are involved in the present synthesis of aspartic acid.

I. II.

$$\begin{array}{c} \text{COOC}_2\text{H}_5 \\ | \\ \text{C(CH}_2\text{COOC}_2\text{H}_5)\text{N} \\ | \\ \text{COOC}_2\text{H}_5 \end{array} \underset{\text{CO}}{\overset{\text{CO}}{\diamondsuit}} \text{C}_6\text{H}_4 \xrightarrow[\text{(HCl)}]{5\ \text{H}_2\text{O}} \text{CO}_2 + 3\ \text{C}_2\text{H}_5\text{OH} + \text{C}_6\text{H}_4\,(\text{COOH})_2 -$$

III.

$$\begin{array}{ccc} \text{COOH} & & \text{COOH} \\ | & & | \\ \text{CHNH}_2 \cdot \text{HCl} & \xrightarrow{\text{Ag}_2\text{O}} & \text{CHNH}_2 \\ | & & | \\ \text{CH}_2 & & \text{CH}_2 \\ | & & | \\ \text{COOH} & & \text{COOH} \\ \text{IV.} & & \text{V.} \end{array}$$

Phthalimidomalonic ester (I) was prepared by the method of Osterberg (10). In preparing sodium phthalimidomalonic ester (II) by the method of Sörensen (11) where absolute alcohol is used as the solvent a 63 per cent yield of impure product was obtained. This is due to the difficulty of completely removing the alcohol from the equilibrium mixture of the sodium compound, phthalimidomalonic ester, and sodium ethylate. When benzene was used as the liquid medium an 83 per cent yield of impure product was obtained. This method is unsatisfactory because the sodium becomes coated with the insoluble sodium phthalimidomalonic ester and thus does not react completely. Dry toluene was found to be an ideal medium for this purpose. The sodium becomes molten in the boiling fluid and quickly reacts to form from 80 to 90 per cent yield of light yellow product which, without further purification, contains the theoretical quantity of nitrogen.

With an 8:1 molal ratio of chloroacetic ester and sodium phthalimidomalonic ester the addition product, ethane-α-phthalimido-α, α, β-tricarboxylic acid triethyl ester (III), was formed as a viscous dark oil. The reaction was complete in $1\frac{3}{4}$ hours as shown by Volhard analyses of the liberated chloride ions (see Fig. 1). After purification and drying a 92 per cent yield of prod-

uct containing almost the theoretical amount of nitrogen was obtained.

Aspartic acid (V) was obtained by the hydrolysis of this oil. Preliminary experiments with absolute ethyl alcohol and sodium
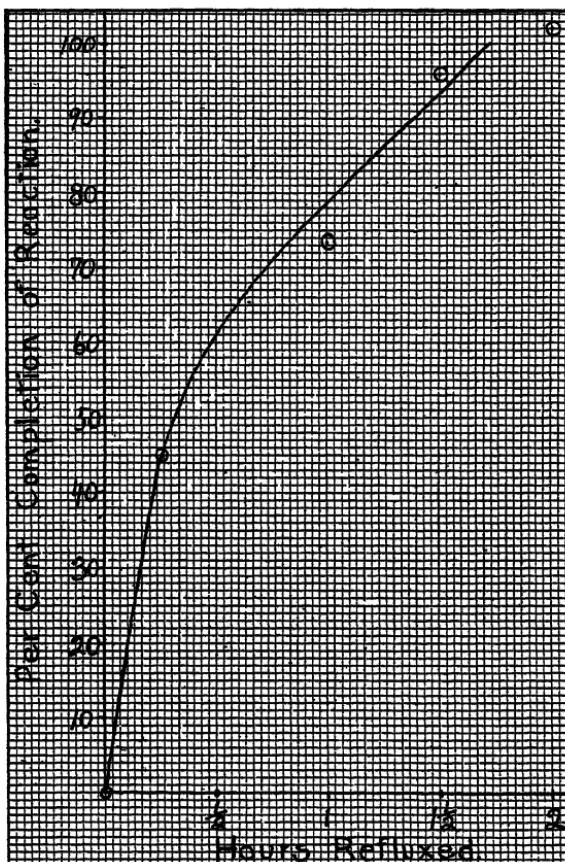
FIG. 1. Volhard titration curve of chloride ions from the reaction of an 8:1 molal ratio of chloroacetic ester and sodium phthalimidomalonic ester. No solvent was used in this reaction.

hydroxide as the hydrolyzing agent were carried out. Here an attempt was made to measure the rate of hydrolysis by means of the liberated carbon dioxide and to obtain aspartic acid in the free state and as its copper salt but the results were inconclusive.

The most satisfactory method of hydrolysis was found to be that with 95 per cent ethyl alcohol and concentrated hydrochloric acid. On the basis of the amino groups, freed during hydrolysis, the reaction went to completion in 24 hours under the experimental conditions (see Fig. 2). Amino nitrogen was measured by the Van Slyke gasometric method. On account of the abnormal blank given by ethyl alcohol it was necessary to remove the latter before making the amino nitrogen determinations.
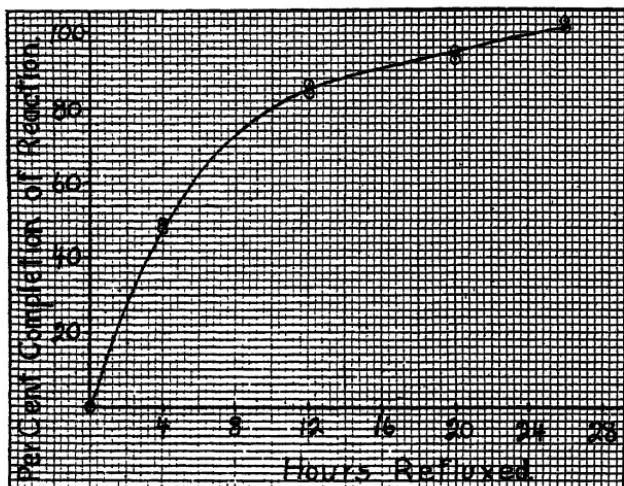
FIG. 2. Curve for amino nitrogen from the decomposition of ethane-α-phthalimido-α,α,β-tricarboxylic acid triethyl ester (III) by ethyl alcohol and an 18:1 molal ratio of concentrated hydrochloric acid. Amino nitrogen was determined by the gasometric method of Van Slyke on solutions from which the ethyl alcohol had been removed by distillation.

After treating the hydrolysate as described in the experimental part a first crop of white aspartic acid crystals was obtained. The yield based on the ethane ester derivative (III) was about 33 per cent. The photomicrographs (shown in Figs. 3 and 4) of these crystals are similar to those of natural aspartic acid as given by Keenan (12).

After one recrystallization the second crop of crystals was white and sweet tasting. That this substance is probably glycine was indicated by subsequent analyses. Experiments are under way

to determine whether glycine results from the decomposition of the addition compound directly or aspartic acid.

A study of the reaction of other halogen compounds with sodium phthalimidomalonic ester was made in connection with the experiments reported here. Since Sörensen has already shown that benzyl chloride, trimethylene bromide, and γ-chlorobutyronitrile react with sodium phthalimidomalonic ester to form products from which phenylalanine (11), proline (13), and aminoadipic acid (14) can be prepared it was expected that β-chloropropionitrile could be used similarly for the synthesis of glutamic acid.

FIG. 3.   FIG. 4.

FIGS. 3 AND 4.   × 80.   Photomicrographs of racemic aspartic acid.

It was found, however, that neither this substance nor ethylene bromide, ethylene chloride, methylene bromide, and β-chloropropionic ester form stable addition products. In all of the experiments the principal product proved to be phthalimidomalonic ester. That reactions had occurred was shown by Volhard analyses which, in some cases, indicated that the halogen had been transformed almost completely into the ionic state. Comparative results of these reactions are shown in Fig. 5.

It is significant that an appreciable quantity of phthalimidomalonic ester also is formed in the preparation of γ-bromopropylphthalimidomalonic ester. Sörensen explains this secondary reaction by assuming that sodium phthalimidomalonic ester ex-

tracts hydrogen bromide from trimethylene bromide with the formation of phthalimidomalonic ester, sodium bromide, and allyl bromide or symmetrical allylene. In the present experiments it is not impossible that analogous reactions may have occurred. In order to show that the procedures used were not the cause of the failures noted, copper prolinate was prepared by Sörensen's method. It was also shown definitely that the glycine,
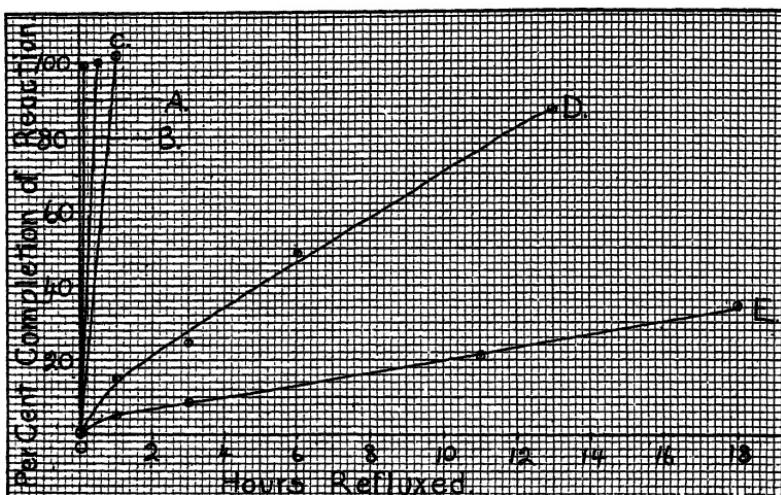
Fig. 5. Volhard titration curves for halogen ions from the reaction of sodium phthalimidomalonic ester with the following halogen compounds; *A*, β-chloropropionic ester, *B*, β-chloropropionitrile, *C*, trimethylene bromide, *D*, ethylene bromide, and *E*, methylene bromide. The following molal ratios of halogen compound to sodium phthalimidomalonic ester were used; *A*, 6:1, *B*, 3.2:1, *C*, 4:1, *D*, 4:1, and *E*, 8:1. No solvent was used in these reactions.

which is formed during the synthesis of proline, results from the decomposition of phthalimidomalonic ester. By the direct alkaline hydrolysis of this ester a 20 per cent yield of glycine was obtained.

<div align="center">EXPERIMENTAL.</div>

Sodium Phthalimidomalonic Ester (*II*).—In a 2 liter three necked flask fitted with a reflux condenser, separatory funnel,

and a mechanical stirrer were placed 400 cc. of toluene (dried over calcium chloride) and 163.5 gm. of phthalimidomalonic ester. After heating the mixture to dissolve the ester 12.3 gm. of purified sodium were added. Freshly cut sodium was purified by heating and shaking it in toluene. The resulting shiny balls were weighed in toluene and then transferred to the reaction mixture. After the mixture was refluxed for 1.5 hours to complete the reaction it was allowed to cool, filtered, washed with 200 cc. of toluene, and dried at 105° for 24 hours. The yield was 148 gm. (85 per cent of the theoretical amount) of light yellow product which decomposed into a dark brown oil at 280°. The product dissolves in cold water giving a yellow solution which gradually hydrolyzes to white, insoluble phthalimidomalonic ester. Hydrolysis is more rapid in boiling water and acid solutions. It is very soluble in methyl alcohol, less so in ethyl and isopropyl alcohol, and is practically insoluble in benzene and toluene.

Calculated for $C_{15}H_{14}O_8NNa$. N, 4.28. Found (Kjeldahl). 4.26, 4.26.

Ethane-α-Phthalimido-α,α,β-Tricarboxylic Acid Triethyl Ester (III).—Chloroacetic ester was prepared according to the method of Conrad (15). From 200 gm. of technical grade chloroacetic acid, 120 gm. of absolute ethyl alcohol, and 25 gm. of concentrated sulfuric acid a yield of 203 gm. (78 per cent of the theoretical amount) of product which boiled at 144–145° was obtained.

50 gm. (0.15 mol) of sodium phthalimidomalonic ester and 112 gm. (0.91 mol) of chloroacetic ester were placed in a 250 cc. flask fitted with an air condenser. The mixture was refluxed on an oil bath at 150° for 1¾ hours (as shown in Fig. 2 this time is approximately that necessary to complete the reaction). The excess chloroacetic ester was removed immediately by distillation *in vacuo,* the dark brown viscous mass extracted with 300 cc. of diethyl ether, and the ether extract evaporated. The yield of heavy residual oil, dried at 45° for 48 hours, was 60 gm. (92 per cent of the theoretical amount).

Calculated for $C_{19}H_{21}O_8N$. N, 3.58. Found (Kjeldahl). 3.60, 3.58.

Aspartic Acid (V).—In a 1 liter flask fitted with a reflux condenser were placed 55 gm. (0.14 mol) of the above addition prod-

uct, 430 cc. of 95 per cent ethyl alcohol, and 250 cc. of concentrated hydrochloric acid. The mixture was refluxed for 27 hours (as shown in Fig. 2 approximately 24 hours were required for the liberation of the contained amino nitrogen in the free amino form).

The resulting homogeneous brown solution was evaporated on a salt water bath. The residue was dried for an hour *in vacuo* over calcium chloride and potassium hydroxide to remove excess hydrochloric acid. The resulting mass, consisting of a crystalline solid and a dark oil, was heated with 50 cc. of water, cooled, filtered, and washed well with water. It was then found that this treatment had dissolved practically all of the aspartic acid as the hydrochloride. Amino nitrogen analyses showed that the undissolved solids and oil (total of 17 gm.) contained only 0.0018 equivalent or 1.3 per cent of the nitrogen originally present in the 55 gm. of addition compound. This residue is probably phthalic acid. The filtrate was then boiled with 1 gm. of decolorizing carbon, cooled, filtered, and the residue washed with 10 cc. of water. This residue contained only a trace of amino nitrogen while 0.125 equivalent was present in the filtrate according to a Volhard titration of chloride ions and 0.131 equivalent by amino nitrogen analysis. Freshly prepared silver oxide from 23.5 gm. (0.138 equivalent) of silver nitrate was stirred into the hot filtrate and after 10 minutes the precipitated silver chloride was filtered from the hot solution. The excess silver was then removed from the filtrate by means of hydrochloric acid. The combined silver residues contained 0.026 equivalent of amino nitrogen. This loss probably is partly due to the formation of insoluble silver aspartate.

The filtrate was evaporated to 100 cc., let cool, and 100 cc. of 95 per cent alcohol added. After 30 minutes crystals began to appear. After 2 days standing in the ice box the almost white crystals were filtered, washed with alcohol, and dried at 45° for 4 days. The yield was 6.2 gm. (33 per cent of the theoretical amount) of product which decomposed from 311–326°. Keimatsu and Kato (9) give 325° as the decomposition temperature of racemic aspartic acid.

Calculated for $C_4H_7O_4N$. N,10.53. Found (Kjeldahl). 10.51, 10.74, 10.62, 10.69.

The crude aspartic acid (4.5 gm.) was dissolved in 100 cc. of boiling water and filtered hot. The crystals resulting from the addition of 200 cc. of 95 per cent alcohol and 3 days standing in the ice box were dried at 105° for 4 hours. The yield was 4 gm. (89 per cent of the weight taken) of white, sour tasting crystals which started to brown at about 325° and were completely decomposed at about 370°.

Calculated for $C_4H_7O_4N$. N, 10.53. Found (Kjeldahl), 10.27, 10.34, 10.27; (Van Slyke), 11.14, 10.84, 10.84.

The mother liquors from the aspartic acid containing 0.064 equivalent of amino nitrogen were evaporated to 25 cc., 50 cc. of 95 per cent alcohol added, and let stand in the ice box for 7 days. A yield of 2.65 gm. of crystals, which upon recrystallization were white and sweet, was obtained. This substance, assumed to be glycine, yielded 25 per cent of the theoretical amount. After this product was dried at 105° for 4 hours it decomposed from 211–221°. It was evidently somewhat impure as low values for nitrogen were obtained by both Kjeldahl and Van Slyke deter-minations.

SUMMARY.

1. A new synthesis of aspartic acid has been described in which a 33 per cent yield of this amino acid has been obtained. Also a product thought to be glycine was isolated from the reaction mixture.

2. Sodium phthalimidomalonic ester, prepared in high yield and high purity by a new method, has been found to react readily with various organic halogen compounds but only in the case of chloroacetic ester and trimethylene bromide were stable addition products formed.

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HEMOGLOBIN MAINTENANCE AND PRODUCTION UPON SYNTHETIC DIETS, INCLUDING MODIFICATIONS IN THE ETHYL XANTHATE AND BIAZZO METHODS FOR COPPER ANALYSIS. I.

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The point of view, developed mainly by Whipple and Robscheit-Robbins (1), that certain foodstuffs possess special virtues as curative agents in experimental secondary anemia has been generally accepted. In recent years great strides have been made in nutritional research by the use of so called synthetic diets, composed in large part of purified food constituents. It seemed pertinent, therefore, to inquire whether a synthetic diet, which has been shown to maintain experimental animals in apparent health, is also a maintenance diet in respect to hemoglobin.

The primary object of this investigation was put in the question: Will animals fed exclusively upon synthetic diets over long periods of time maintain hemoglobin at a normal level? During the course of this work there were published the very interesting papers of Hart and his associates (2) upon the importance of the salts of copper in the cure of severe anemia in rats due to an exclusive milk diet. While not losing sight of the original purpose of the problem, we deemed it advisable to gain, if possible, further information concerning the significance of copper in hemoglobin formation.

About a year ago a preliminary report (3) was made upon the early part of this work. The copper analyses, which were the basis for the interpretation of the results, were by the ethyl xanthate method (4). Simultaneously with the publication of this note, Elvehjem and Lindow (5) gave up the use of the ethyl xanthate method and introduced a slightly modified Biazzo technique for

the determination of copper. The inability to check the earlier copper analyses and the wide divergence in the analytical results obtained by the ethyl xanthate and Biazzo methods made it probable that one or both methods were not sufficiently accurate for the determination of exceedingly small amounts of copper, although the methods are probably accurate enough for the larger quantities determined by the original investigators.

A critical study of the Biazzo and ethyl xanthate methods has accordingly been made and has resulted in the modification of each to insure accuracy in the determination of minute amounts of copper.

Methods.

Both dogs and rats have been used in this study. All animals, including the rats, were kept in individual cages. The dogs were adult animals of a mongrel terrier type, 7 to 13 kilos in weight. The rats were "skin parasite-free," most of them of the Wistar Institute Experimental Colony strain, although piebald rats (Wisconsin strain) were also used. They were kept in a separate room, under ideal laboratory conditions. The possibility of food contamination was eliminated. Copper-free distilled water (redistilled from glass) was provided *ad libitum*. At an early age (22 to 30 days), the rats were placed upon a diet of whole milk. After the development of a rather severe anemia, they were transferred to various synthetic rations and the effect on hemoglobin production was periodically determined.

The diets used were:

(*a*) The Karr-Cowgill (6) synthetic ration. This diet was employed in the experiments upon dogs. It was fed on the basis of 16 gm. per kilo of body weight, with the daily addition of 0.6 gm. of of Vitavose (a source of the antineuritic vitamin) per kilo.

(*b*) The Karr-Cowgill diet with copper sulfate added. Copper sulfate was added to the Karr-Cowgill diet, so that the level of copper fed was raised to 1.3 mg. per kilo of body weight. In order to avoid possible incompatability between the copper sulfate and potassium iodide in the salt ration (*i.e.* the formation of insoluble CuI), the diet containing the copper sulfate was fed 6 days a week and on the 7th day the weekly quota of potassium iodide was administered in a portion of Karr-Cowgill ration to which no copper

sulfate had been added. The same precaution was observed in the experiments upon rats, when the diet rich in copper was used.

(c) Low copper Diet 1 for rats. This ration was modelled after one of Osborne and Mendel's (7) diets upon which rats grew unusually well. The composition of the diet appears in Table I. When fed *ad libitum*, 7 to 10 gm. of this ration were consumed daily by a rat.

(d) Low copper Diet 2 for rats, which is shown in Table II.

(e) Low copper Diet 3 for rats. This was an incomplete ration, essentially similar to low copper Diet 2 except for the omission

TABLE I.

Low Copper Diet 1.

	Approximate amount per rat per day.		Calories per rat per day.
	gm.	*per cent*	
Albumin* (egg)............................	3.5	35	14.0
Starch (corn).............................	4.8	48	19.2
Lard.....................................	0.9	9	8.1
Cod liver oil.............................	0.4	4	3.6
Salt (mixture)†..........................	0.4	4	
Total...................................	10.0	100	44.9
Addendum (source of vitamin B).			
Harris yeast concentrate.................	0.12	1.2	
Brewers' yeast..........................	0.30	3.0	

* The albumin was coagulated by heat in order to avoid diarrhea in the rats (8).

† The Cowgill salt mixture (6) was used.

of cod liver oil and Harris yeast concentrate, making the diet very low in the accessory food factors.

(f) A high copper synthetic ration for rats. Copper sulfate was added to the low copper Diet 1 so that approximately 0.05 mg. of copper per rat per day was furnished.

The salt mixture used in low copper Diet 1 for rats and also in the dog rations is described in Table III of Cowgill's paper (6). The various salts used were of the highest purity available on the market.

There are apparently no exact data upon what constitutes a normal hemoglobin level. ±15 per cent of the Haldane standard

(oxygen capacity of 18.5 volumes per cent equivalent to 13.8 gm. of hemoglobin per 100 cc. of blood or 100 per cent hemoglobin) appears to be an acceptable figure for the normal variation in the dog and rat. The level of hemoglobin has been shown to vary about 24.6 per cent during the first 150 days of the life of a rat (9). Earlier in the study the Cohen and Smith (10) method was used for the determination of hemoglobin. Later, the Newcomer method (11) was adopted for routine determinations after complete restandardization of the colored plate for different hemoglobin concentrations. This was based upon accurate Van Slyke and Stadie (12)

TABLE II.

Low Copper Diet 2.

	Approximate amount per rat per day.		Calories per rat per day.
	gm.	*per cent*	
Albumin* (coagulated)......................	4.1	41	16.4
Dextrin*...................................	5.1	51	20.4
Lard.......................................	0.3	3	2.7
Cod liver oil..............................	0.2	2	1.8
Sodium chloride...........................	0.2	2	
$Fe_2(SO_4)_3 \cdot 5H_2O$†			
Harris yeast concentrate..................	0.1	1	
Total.................................	10.0	100	41.3

* The albumin and dextrin were of the highest purity available on the market.

† The iron salt was fed at a level of 0.00088 gm. per day, corresponding to 0.2 mg. of iron, and was of the highest purity available.

oxygen capacity determinations on whole blood, with calibrated apparatus, and spectrophotometric determinations of acid hematin solutions. From time to time during the course of the experiments the values for hemoglobin obtained by means of the "calibrated" Newcomer plate were again checked by Van Slyke and spectrophotometric determinations of the same blood samples.

Dog blood was obtained as a routine from the ear veins. Consecutive, duplicable small samples were readily drawn in this manner. When larger quantities were required cardiac puncture was the procedure adopted. An ideal method for obtaining

TABLE III.

Determination by Biazzo Method of Copper in Solutions Containing Known Amounts of Copper and Iron.

Amount of copper* present.	Amount of iron* present.	Amount of copper found.	Error in determina-tion.	Remarks.
mg.	*mg.*	*mg.*	*per cent*	
0.010	0.005	0.0215?	+115.0	Reading very poor.
0.025	0.000	0.0249†	−0.4	
0.025	0.005	0.0392†	+56.8	
0.025	0.010	0.0352	+40.8	
0.025	0.015	0.0396†	+58.3	
0.025	0.020	0.0386	+54.4	
0.025	0.025	0.0418	+67.2	Slightly off tint.
0.025	0.030	0.0449†	+79.5	" " "
0.025	0.040	0.0388	+55.2	" " "
0.025	0.050	0.0522†	+108.8	" " "
0.050	0.000	0.0500	0.0	
0.050	0.005	0.0604	+20.8	
0.050	0.025	0.0617	+23.4	
0.050	0.025	0.0598	+19.6	Solution boiled to dryness and treated with acids, etc.
0.050	0.050	0.0613†	+22.6	
0.050	0.100	0.0627†	+25.4	
0.250	0.000	0.2509	+0.4	
0.250	0.005	0.2890	+15.6	
0.250	0.010	0.2840	+13.6	
0.250	0.015	0.2810	+12.4	
0.250	0.020	0.2895	+15.8	Slightly off color.
0.250	0.025	0.2750	+10.0	
0.250	0.030	0.2820	+12.8	
0.250	0.040	0.2820	+12.8	
0.250	0.500	0.5950?	+138.0	Very poor comparison; yel-lowish.

* Kahlbaum crystalline $CuSO_4$ was used in making up the standard copper and copper-iron solutions. The sources of the iron in the copper-iron solutions were Kahlbaum and Powers-Weightman-Rosengarten analytical $Fe_2(SO_4)_3$. Copper-iron solutions were also prepared from iron purified according to the method of Waddell, Steenbock, and Hart (13). The results obtained with these solutions were identical with the above.

† Average of readings with the standard copper solution set 10 and 20 mm. in a Duboscq micro colorimeter. In all other cases the standard solution was set at 10 mm. In all instances the amount of copper in the standard was identical with that in the copper-iron solution.

small blood samples from rats is wanting. The usual technique of clipping the tail was used.

Copper was determined by the methods which will be described under *"Experimental."*

In studying the Biazzo method it was discovered that iron interfered with the determination of copper. When the same acidity and technique were employed as in the method for copper (5), the addition of 1 cc. of 10 per cent KCNS to $Fe_2(SO_4)_3$ solutions containing 0.01 to 0.05 mg. of the Fe^{+++} produced, as might be expected, pink-tinted solutions. The addition of 10 drops of pyridine to these solutions practically decolorized them. The solutions were then extracted by shaking each with 5 cc. of chloroform. In every instance the chloroform layer became as highly colored (pink) as the original solution before the addition of pyridine. When 0.5 mg. of Fe^{+++} was used, the deep red color with KCNS was changed to a slight pink (not completely decolorized) after the addition of pyridine, while the chloroform layer was once again deep red.

The color of the copper-thiocyanate-pyridine compound, supposedly formed in the Biazzo determination, is yellow-green. The color of dilute solutions of $Fe(CNS)_3$ is pinkish. However, when the Biazzo method was used to determine small amounts of copper (0.02 to 0.25 mg.) in the presence of minute amounts of iron (0.005 to 0.1 mg. of Fe^{+++}), the amount of color development was greater than in iron-free solutions containing the same quantities of copper. In the above copper-iron solutions the color of the chloroform extract was not sufficiently different from the color of the copper-thiocyanate-pyridine compound to be colorimetrically distinguishable. Only when larger quantities of Fe^{+++} were present was the color sufficiently different to make colorimetric comparison impossible. Table III indicates that the presence of minute quantities of Fe^{+++} interferes very seriously with the determination of copper by the Biazzo method.

In searching for a method to remove the interference of iron in the determination of copper, it was found, through a suggestion in a paper by Warburg (14), that sodium pyrophosphate could be effectively employed. 3 to 10 drops of a 4 per cent solution were

sufficient for the quantities of iron usually encountered in this work. In copper-iron solutions such as shown in Table III several drops of 4 per cent $Na_4P_2O_7$ (added either before the addition of KCNS and pyridine or after the addition of KCNS) completely removed the error due to iron, so that theoretical results for copper were obtained. This was true even in the case of the solution containing 0.25 mg. of copper and 0.5 mg. of iron. In order to maintain comparable conditions the same number of drops of pyrophosphate solution were, of course, added to the known copper solution as to the unknown.

When $Na_4P_2O_7$ is added to copper-iron solutions $Fe_4(P_2O_7)_3$ and $Cu_2P_2O_7$ are formed. The former is an insoluble precipitate, while the latter remains in solution with the intensification of blue color. $Fe_4(P_2O_7)_3$ does not react with such ions as CNS^-, while $Cu_2P_2O_7$ does. When relatively large quantities of Fe^{+++} were present, a slight turbidity (due to the Fe precipitate) was produced upon the addition of sodium pyrophosphate. This, however, did not interfere with the quantitative extraction by means of chloroform of the copper-thiocyanate-pyridine compound.

In the Biazzo method it was also found advisable to employ standards which contained not less than 0.015 mg. of copper. This was necessitated by the fact that it was difficult to obtain non-turbid chloroform extracts from standard solutions containing only 0.01 mg. of copper. It should be noted that the turbidity seemed to be related to the concentration of metallic salts, since the chloroform extract could be clarified by the addition of solutions containing 0.005 mg. of Cu^{++} or Fe^{+++}.

In using the potassium ethyl xanthate method for the determination of small amounts of copper, it was found important to adjust the pH of both the unkown and standard solutions accurately. The directions given by Scott (4) call for the use of "neutral or only very slightly acid" solutions. It was readily demonstrated that the color development upon the addition of potassium ethyl xanthate to a standard copper solution at different pH levels, from. pH 1 to 7, was appreciably less at the two ends of the pH range than at the middle. The maximum color development was at pH 3.8. This acidity could be insured by using 10 cc. of phthalate buffer of pH 3.8 (15) without interfering with the determination. For eliminating the interference due to small amounts of iron,

TABLE IV.

Copper Analyses upon Milk.

Milk, 200 cc.*	Amount of copper found.	Method of analysis.
	mg.	
	0.080	Unmodified ethyl xanthate.
	0.070	
	0.065	Ethyl xanthate; pH 3.8 insured by
	0.065	addition of buffer.
Plus 0.05 mg. Fe.	0.065	
" 0.10 " "	·0.070	
" 0.20 " "	0.090	
" 0.01 " Cu.	0.080	
	0.050	Biazzo method as modified by Elve-
	0.055	hjem and Lindow (5); copper precipi-
	0.050	tated as sulfide with H_2S.
Plus 0.05 mg. Fe.	0.055	
" 0.01 " Cu.	0.045	
" 0.05 " "	0.080	
" 0.05 " Fe.	0.060	Ethyl xanthate; pH 3.8 insured,
" 0.10 " "	0.065	$Na_4P_2O_7$ used.
" 0.20 " "	0.060	
" 0.05 " "	0.022	Biazzo method without use of H_2S
" 0.20 " "	0.056	(unmodified).
" 0.20 " "	0.036	Biazzo method without use of H_2S,
		but $Na_4P_2O_7$ added.

* The milk was Walker-Gordon certified. Before the samples were evaporated to dryness on a steam bath, the caseinogen was precipitated at the isoelectric point. The whey and protein precipitate were then "dried" together without loss of material, since boiling over was effectively prevented. The ashing was in quartz or platinum crucibles in an electric muffle at dull red heat. Although several different quarts of milk were used in these analyses, the correct interpretation of results does not demand taking this factor into consideration. This is so because of the constancy in the copper content of milk and because care was taken to run analyses by different methods upon samples from the same quart of milk.

TABLE V.
Copper Analyses upon Synthetic Diet.

Low copper Diet 1, 10 gm.	Amount of copper found.	Method of analysis.
	mg.	
	0.0100	Unmodified ethyl xanthate.
	0.0150	
	<0.0100	
	0.0187	Unmodified Biazzo method.
	0.0260	
	0.0200	
*	0.0137	
	0.0122	
	0.0210	
	0.0230†	
Without iron salt.	0.0080	
‡	0.0150	Ethyl xanthate; pH 3.8 insured by addition of buffer.
‡	>0.0050	Ethyl xanthate; pH 3.8 insured, $Na_4P_2O_7$ added.
	<0.0100	
*	0.0043	Biazzo method with $Na_4P_2O_7$.
	0.0068	
	0.0071	
Without iron salt.	0.0075	
Plus 0.01 mg. Cu.	0.0181	
" 0.01 " "	0.0110	Biazzo method with Zn and H_2S (5).

* The ashes of these samples were dissolved together in HCl. The final solution was divided in two equal parts and the copper in each determined as indicated.

† This analysis was kindly carried out by Professor Hart. The analyses of 0.015 and <0.01 mg. of copper by the unmodified xanthate method and 0.0187 and 0.026 mg. by the unmodified Biazzo technique were upon the batch of diet, a sample of which was sent to Professor Hart. All the other analyses were upon another batch of diet, which was made with the same materials. All the organic constituents except the lard and cod liver oil were thoroughly mixed together and divided into two portions. To one (the larger portion) was added the salt mixture containing iron, to the other a salt mixture composed of the same salts but with the iron salt omitted. After thorough mixing, an appropriate quantity of melted lard and cod liver oil was added to each portion.

‡ The ashes of these samples were also taken up together in HCl, the final solution being divided in two equal parts.

sodium pyrophosphate was found just as effective in the xanthate as in the Biazzo method. However, when large quantities of iron were present, the precipitate of iron pyrophosphate rendered the solution quite turbid. It was found difficult completely to clarify the solution by filtration. In this event the copper may be separated from the iron by the use of "more positive" metals such as aluminum or zinc.

Tables IV and V show the results of copper analyses upon milk and upon a synthetic ration, low copper Diet 1 for rats (described in Table I). The following points are brought out.

(a) The analyses upon milk by the ethyl xanthate method are very consistent. When pH 3.8 is insured by the use of phthalate buffer solution, the amounts of copper are about 13 per cent lower than by the unmodified method. The figures by the Biazzo technique (as used by Elvehjem and Lindow (5) for copper in milk) are still lower, by about 15 per cent. It may also be pointed out that all the analytical results are appreciably higher (60 to 110 per cent) than the average of Elvehjem, Steenbock, and Hart (16).

(b) As much as 0.1 mg. of iron (as $Fe_2(SO_4)_3$) can be added to 200 cc. of milk without interfering with the determination of copper. This is proved by the fact that the analyses by the xanthate method at pH 3.8 are practically identical with those by the same method, when, besides the buffer, $Na_4P_2O_7$ is used. This is in marked contrast with the effect of iron in pure copper-iron solutions. However, when 0.2 mg. (about 3 times as much as the copper present) of iron is added to 200 cc. of milk, the error in determination is appreciable unless sodium pyrophosphate is employed.

(c) The recovery (in the analysis) of 0.01 mg. of copper added as $CuSO_4$ to 200 cc. of milk is complete, when the analysis is by the ethyl xanthate method with pH 3.8 insured. On the other hand, 0.01 mg. of added copper was not recovered by the Elvehjem and Lindow (5) modification of the Biazzo technique (in which the copper is precipitated as the sulfide by means of H_2S); when 0.05 mg. of copper was added the recovery by this technique was only 60 per cent.

(d) In the analysis of milk by the unmodified Biazzo method, phosphates are precipitated upon neutralization and are not dissolved after the addition of acetic acid. As found by Elvehjem

and Lindow, the precipitate probably prevents the complete extraction of the copper-thiocyanate-pyridine compound. Although these figures are low they show that the addition of 0.2 mg. of iron to the milk here also raised the analytical result and that $Na_4P_2O_7$ effectively corrected the error due to the presence of iron. The use of pyrophosphate, however, has no influence upon the error due to the precipitation of phosphates. Under these circumstances it appears that the Biazzo technique cannot be relied upon for the determination of copper in milk.

(*e*) The figures for the analyses (Table V) of samples of low copper Diet 1 by the unmodified xanthate and unmodified Biazzo methods are very variable (0.01 to 0.026 mg.). The writers have taken the liberty to include an analysis of 0.023 mg., which was furnished by Professor Hart.[1] It may be seen that the discrepancy between this figure and some of the others in Table V is small in those cases where the same method of analysis was used.

(*f*) When $Na_4P_2O_7$ is used very consistent results are obtained and the analyses (about 0.008 mg. of copper) by the xanthate and Biazzo methods agree. The results are considerably lower than by the unmodified methods. When the iron salt is not added to the diet, so that the only source of iron is in the adventitious contamination of the other constituents, the analyses by the unmodified Biazzo and by the Biazzo-pyrophosphate technique are practically identical (0.008 and 0.0075 mg.).

(*g*) With the Biazzo-pyrophosphate method, 0.01 mg. of copper added as $CuSO_4$ to 10 gm. of diet is recovered completely. When Zn and H_2S (recommended for the determination of copper in the presence of excess iron (5)) are used with the Biazzo method the recovery of 0.01 mg. of added copper is very poor.

Five dogs (the data upon four of which are shown in Chart I), fed upon the Karr-Cowgill diet for periods of 6 months to nearly 4 years, maintained their hemoglobin at a fairly constant normal

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[1] A number of samples of diet were sent to Professor Hart. One of these, a large quantity (about 300 gm.) of low copper Diet 1, was freshly prepared. This is the material upon which Hart obtained the figure of 0.023 mg. of copper per 10 gm. of diet and was the only sample upon which analyses by both groups of workers were available. This figure was not quoted by the Wisconsin investigators (17) in a short note of criticism of the preliminary report upon the early part of this work (3). The regrettable impression, contrary to fact, was also created that this diet contained casein.

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level from week to week. Dog 1 in Chart I merits some attention. It is a myxedematous animal, which since puppyhood (2 years before the onset of this particular study) had been on the Karr-Cowgill diet. This dog was slightly anemic (probably related to absence of the thyroid glands) and remained so in spite of nutritional procedures. Dog 4 was bled approximately 30 per cent of its calculated blood volume. Regeneration of this single hemor-

CHART I. Hemoglobin maintenance in dogs on Cowgill's diet.

rhage was gradual but effective on the synthetic ration. Chart I also brings out the fact that the addition of copper to the Karr-Cowgill diet has failed to make it any more efficient in respect to hemoglobin maintenance.

The Karr-Cowgill diet was analyzed for copper by the unmodified xanthate method with the following results. (1) 16 gm. of diet (the quantity fed per kilo of body weight) was found to contain 0.052 mg. of copper. (2) The analysis for copper in the var-

ious constituents of this ration revealed surprizingly that the individual salts had no copper contamination which could be detected by the unmodified ethyl xanthate method. (3) The amount of copper present in casein (used as a source of protein) was 0.0454 mg. in 16 gm. of diet. (4) The amount of copper present in Vitavose (source of vitamin B) was 0.009 mg. in 16 gm. of diet. (5) The other constituents appeared to be copper-free when the amounts fed per kilo of dog were analyzed.

From the above data it is obvious that the copper in the casein and Vitavose accounts for the copper in the total diet. The quest for a source of protein which would prove "copper-free" has proved successful with the resulting construction of low copper Diet 1 (described under *"Methods"*). Coagulated albumin is used as the protein in this diet. The water used in the coagulation (as well as the drinking water for rats and water used in analyses) was redistilled in glassware. This was necessitated by the fact that the water obtained from a new still in the laboratory was found to contain about 0.6 mg. of copper per liter (analyses upon 100 cc. samples by the unmodified xanthate method). It is of interest to note that this gross copper contamination occurred in spite of the fact that the tank was tin plated and the pipe lines of block tin. It is curious that water from an old still and tank in our former laboratory was practically copper-free by the same method of analysis. Harris yeast concentrate and brewers' yeast supply the water-soluble vitamins. The analyses for the copper content of this diet have been given in Table V. The amount of iron present in 10 gm. was found to be 0.612 mg. by a slightly modified Kennedy method (18). This figure is somewhat higher than that of 0.588 mg. (by the Elvehjem and Hart method (19) kindly furnished by Hart), but is lower than the theoretical, on the assumption that ferric citrate contains 16.6 per cent of iron. In this synthetic ration the main source of copper proved to be in the yeast concentrate and brewers' yeast, particularly the latter. As has already been mentioned, the amount of diet consumed daily by a rat was somewhat less than 10 gm. In a number of instances where careful records were kept the daily consumption of an 80 to 150 gm. rat was 9.4 to 9.6 gm. when fed *ad libitum*. Upon this quantity the rats grew excellently.

Another diet, low copper Diet 2, was prepared. In this diet

Harris yeast concentrate was used as the sole source of water-soluble vitamins and sodium chloride and ferric sulfate were used in place of the rather complex salt mixture. This diet was analyzed and found to contain, per 10 gm., 0.0019 mg. of copper (an average of eight analyses, three of which were 0.000 mg. of copper, while two were 0.004 and 0.005 mg. respectively). The analysis for iron in 10 gm. of this diet was 0.26 mg.
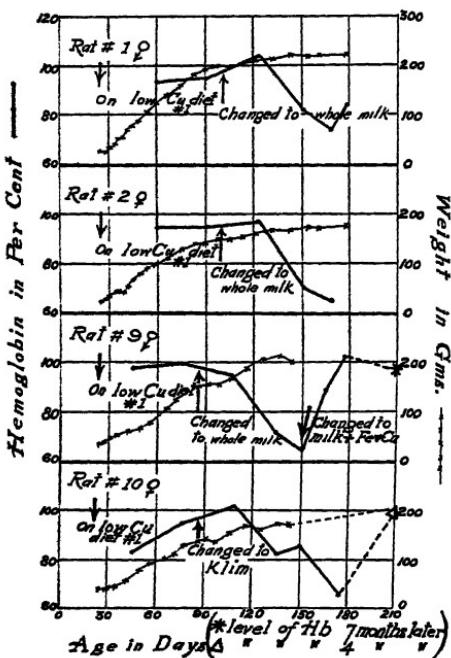
CHART II. Effect on hemoglobin production of changing to milk from low copper Diet 1.

The amount of copper in 10 gm. of low copper Diet 3 (same as Diet 2, but with cod liver oil and Harris yeast concentrate omitted) was too small to determine by the modified Biazzo method. The albumin used in Diet 3 was coagulated by means of ethyl alcohol. This procedure was simpler and more efficient than coagulation by heat and yielded an excellent product. The amounts of synthetic Diets 2 and 3 consumed by a rat per day were somewhat less than

10 gm. The quantity of iron present in low copper Diet 3 was 0.23 mg. per 10 gm. of diet.

The copper analyses upon milk have already been given in Table III. Although young rats consume smaller quantities of milk, the quantity of milk consumed by a 100 to 150 gm. rat was determined to be about 50 cc. per day. After the rats had been on the milk for some time a mild diarrhea almost invariably developed
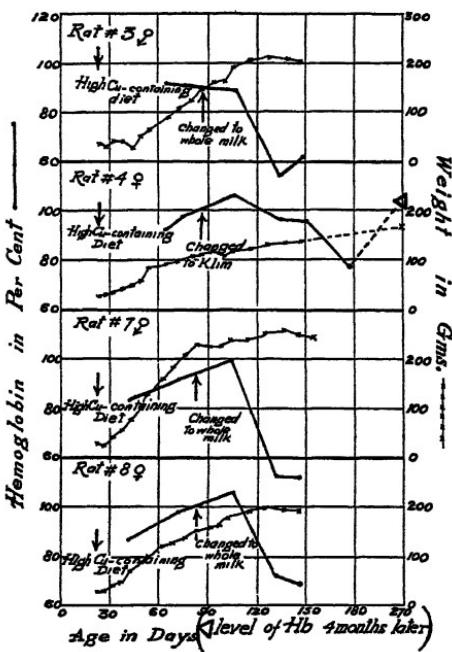
CHART III. Effect on hemoglobin production of changing to milk from high copper diet.

and the consumption of milk was reduced to as low as 20 cc. per day. From our analyses by the modified ethyl xanthate method it may be calculated that, during the period of active milk consumption, the copper intake per rat per day was approximately 0.016 mg.

Several rats were placed on a powdered milk (Klim) diet. This substance was analyzed by the unmodified xanthate method and found to contain 1.70 mg. of copper per kilo. Approximately

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8.0 gm. of this diet were consumed per day per 150 gm. rat, or a daily copper consumption of 0.0136 mg.
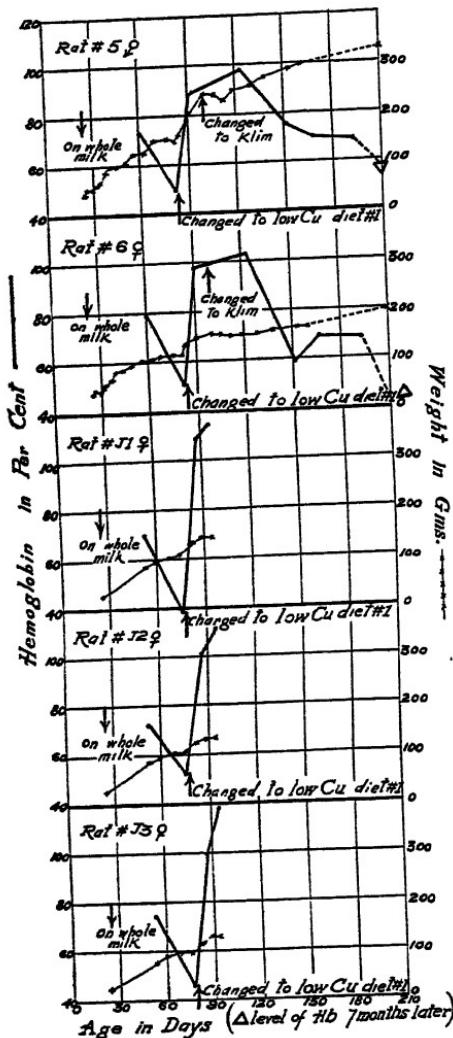
CHART IV. Hemoglobin production on low copper Diet 1.

Chart II shows data from a group of rats upon the low copper Diet 1. The growth of these animals and hemoglobin mainte-nance are entirely satisfactory.

Chart III graphically represents the hemoglobin determinations upon a group of rats on the high copper synthetic ration. It is evident that the hemoglobin levels of these animals are no higher than those shown in Chart II. The growth of this group is also satisfactory.
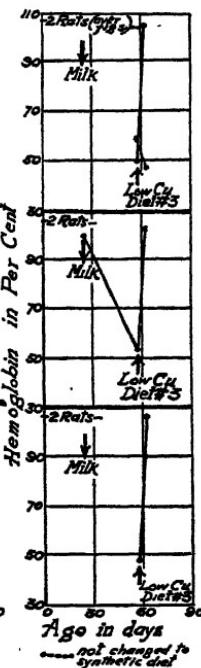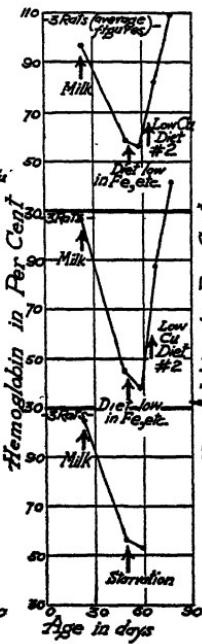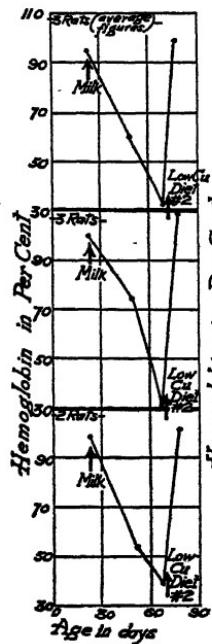
CHART V.　CHART VI.　CHART VII.

CHARTS V TO VII. Hemoglobin production upon low copper Diets 2 and 3.

Charts IV to VII present data upon other groups of rats. On a diet of cow's whole milk they grew fairly well. The severity of the anemia that these rats developed was notable. When the animals had reached a rather low level of hemoglobin they were transferred to the low copper synthetic rations. The rapid, complete recovery from the anemia upon all the synthetic diets was most striking. Chart VI indicates that anemic rats could not be cured upon a diet which was lacking in water-soluble vitamins and in iron, but when

Harris yeast concentrate and ferric sulfate were added, the anemia was promptly cured.

Some added observations should be noted. In Charts II and III it may be seen that some rats which have been on the low copper Diet 1 and some which have been on the high copper-containing ration (same as Diet 1, but with $CuSO_4$ added so that the level fed was about 0.045 mg. of copper) have been placed upon milk or Klim (milk powder). After an interval all the rats became anemic. The rats which have had copper in their dietary seem, if anything, to have become more anemic than those on the low copper diet. This is particularly noteworthy because analytical data (which the writers have obtained upon dogs and which Lindow and coworkers (20) have presented for rats) indicated that a great deal of the copper fed had been retained by the animal. The low-grade anemia upon Klim is also worthy of note.

<div align="center">DISCUSSION.</div>

The data which have been presented upon the determination of small amounts of copper indicate, contrary to the findings of Elvehjem and Lindow (5),-that the Biazzo method is extremely sensitive to minute amounts of iron. The variability of copper analyses upon synthetic Diet 1 by the unmodified methods was probably due to the presence of both iron and phosphates in this material. When the elimination of iron was insured the analyses became quite constant. It was originally shown by Berzelius (21) as well as recently by Warburg (14) that pyrophosphates are produced when substances such as Na_2HPO_4 are heated at dull red heat. The formation of ferric pyrophosphate may be readily demonstrated by evaporating to dryness upon a hot plate a dilute, lightly brown-tinted ferric chloride solution containing Na_2HPO_4. When the dry sediment is dissolved in water, a colorless solution is obtained. This solution when heated with acid once again assumes a brownish tint. It may be assumed that such a reaction would occur during the ashing of milk or the synthetic diets. The quantities of pyrophosphate produced might well be sufficient automatically to eliminate the interference of iron. However, the ash is taken up in 1:1 HCl and evaporated to dryness by heating (Biazzo method). In view of the findings of Lohmann and Eggle-

ton and Eggleton (22), there can be no doubt that a great deal or all of the pyrophosphate which had been formed would be hydrolyzed at this stage of the method.   One, therefore, cannot depend upon the presence of phosphates in biological materials completely to eliminate the error due to iron, and the addition of sodium pyrophosphate in the Biazzo method (before the addition of pyridine) becomes essential.   The following points, however, indicate that phosphates do play a rôle in the analytical findings.   (a) As much as 0.1 mg. of iron (as $Fe_2(SO_4)_3$) could be added to milk which is very rich in phosphates without apparently interfering with the determination of copper.   (b) The interference of iron was not as pronounced in the analysis of the synthetic diet as in the case of pure copper-iron solutions.   (c) In low copper Diets 2 and 3 in which phosphates were not added to the diet the interference of iron was particularly pronounced, although the quantity present was far smaller than in low copper Diet 1.   In these instances the interference of iron was effectively removed by adding the pyrophosphate solution drop by drop until the color produced after the addition of KCNS was completely dispelled.   Without the use of pyrophosphate the Biazzo determination would have been impossible.

The milk upon which rats become anemic contains copper.   In the interpretation of the rôle of copper in anemia it is essential to establish definitely the amount of copper in the milk.   The early analyses of Hart (2) and other investigators (23) indicated that milk contained 0.4 to 0.5 mg. of copper per liter.   It appeared unreasonable (16) that an anemia which was produced upon a copper consumption of 0.01 to 0.017 mg. per day should be cured by the addition of only 0.005 mg. of copper.   The analyses by the Biazzo-H_2S technique, however, indicted that there was only 0.15 mg. of copper per liter of milk, or 0.005 mg. of copper in the quantity consumed by a rat per day.   Data have been presented which indicate that the lower figures by the Biazzo-H_2S technique are probably due to loss of copper during the determination.   This view is in accord with the recent work of Supplee and fellow workers (24), whose analyses (0.5 to 0.75 mg. per liter) by the ethyl xanthate method agree with those which Guérithault (25) has obtained with the ferrocyanide method.

The copper content of the coagulated and dried egg albumin used

by the writers was too low per 5 gm. to be determined by the modified xanthate and modified Biazzo methods. Neither Guérithault (25) nor McHargue (26) was able to determine the copper in egg white. Elvehjem, Kemmerer, Hart, and Halpin (27) claim that dried egg white contains 0.0056 mg. of copper per gm. Guérithault's findings are significant because an independent method of analysis was employed and because his copper analyses upon biological materials were in most instances somewhat higher than those of Elvehjem and Hart and coworkers (28).

In the experiments described above, rats were cured of their nutritional anemia with remarkable rapidity when fed synthetic rations that contained quantities of copper, in one case of the same order of magnitude as that found by Hart and associates in milk and in the other cases quantities definitely less than Hart's figure of 0.005 mg. In low copper Diets 2 and 3 the amount of iron present was a little more than 0.2 mg. per rat per day. Hence the possible argument that in the presence of optimal quantities of iron (0.6 mg.), smaller quantities of copper are needed, becomes untenable. It should also be noted that in many instances the same iron salt has been added to milk so as to furnish 0.2 mg. of iron per rat per day. This quantity of iron was entirely insufficient for the recovery of the anemic rats.

If the milk were copper-free, the problem would be far simpler. One could then definitely assume that copper is a specific supplement to iron in hemoglobin building. Since, however, anemia-producing milk contains copper and a cure of the anemia has been accomplished by synthetic diets which are lower in copper, in this type of experiment the specificity of copper, or even its necessity, becomes highly questionable.

Although confirmatory evidence has been offered in support of the unique importance of copper in hemoglobin production (29), the idea that many catalytic metals besides copper may have stimulating properties in regard to blood formation has been gaining ground recently (30). Manganese, particularly, has come to the front as important in this connection (Titus, Cave, and Hughes (31), Mitchell and Miller (32)). Beard and Myers (30) claim that iron alone is just as effective as iron plus copper, so that in this regard the nutritional anemia of the rat is no different than the anemia due to hemorrhage in the dog (33). The Wisconsin

investigators have not been able to demonstrate unequivocally the importance of manganese and eleven other metals, although their figures for the regeneration of hemoglobin with 0.001 mg. of arsenic are as good as some of those reported with 0.005 mg. of copper (34). Hart (13) considers the contamination of salts of iron with copper as one of the possible causes of the finding that the addition of iron alone to the milk was sufficient to cure the anemia. Elvehjem and Lindow (5) have given figures for the amounts of copper present in various commercial iron salts. The two most contaminated salts cited contained respectively 0.044 and 0.043 mg. of copper per gm. of iron salt. The latter analysis was upon a sample of ferric citrate. If one assumes the formula $FeC_6H_5O_7 \cdot 5H_2O$ for this salt, or a content of about 16.6 per cent of iron, one may calculate that when ferric citrate is used to furnish 0.5 mg. of iron (more than sufficient for regeneration when impure iron salts are used) a little more than 0.0001 mg. of copper is administered inadvertently. Assuming the correctness of Hart's low figure of 0.005 mg. of copper in the milk fed per day and that copper is essential for hemoglobin production, one encounters the incredible situation of a severe anemia which cannot be cured when 0.005 mg. of copper and 0.5 mg. of iron are present, but is promptly cured when 0.0051 mg. of copper and 0.5 mg. of iron are available. Under these circumstances one wonders what interpretation should be placed upon the finding (13) that when "purified" iron was fed at the level of 2 mg. no additional copper was necessary for hemoglobin formation. Is it justifiable to assume that the purified iron was not pure enough? The situation in respect to feeding impure iron salts becomes even more puzzling when one recalls that an unequivocal cure of anemia was not obtained unless at least 0.005 mg. of copper was added to the milk (34).

It is interesting to note that Hart and associates (35) have themselves presumably discovered a species (the pig) in which it is difficult to demonstrate the importance of copper. Although all their experiments indicate that iron alone suffices to cure the anemia of young pigs, the Wisconsin workers feel that somehow copper must have been obtained by the animals or else that the pig needs less copper than the rat.

One might speculate as to the exact cause of the anemia of rats

upon a diet of cow's milk. For many years (36) the low iron content of milk has been thought to account fully for the anemia which develops when infants are kept exclusively upon milk for longer than the normal lactation period. A number of investigators (37, 38) have shown that the milk of one species is quite different in composition from that of another. This work suggests the possibility that cow's milk may be poor in type for the rat. Rats living exclusively on a milk diet suffer from diarrhea. Copper is an astringent (39) and whether it acts in some beneficial manner locally in the gut of the rat on a milk diet cannot be said at present. At least one species, the rabbit, has been reported to develop serious pathological lesions of the intestine on a diet of cow's milk (40). Histological sections have been made[2] of various regions of the intestines of rats which have died of severe anemia. No evidence of serious pathological lesions has been found. A number of suggestions (20) have been made as to the manner in which copper may act as a stimulant for hemoglobin production, but the experiments, thus far, have been entirely inconclusive.

The finding that rapid hemoglobin production can be brought about without feeding of vitamins is of interest in that it indicates that in this regard the nutritional anemia in the rat is no different from the anemia due to hemorrhage in the same species (McCay (41) and Cartland and Koch (42)).

The speed with which rats regenerate hemoglobin upon the synthetic diets is noteworthy. The hemoglobin was doubled in 7 days and often tripled in 10 to 14 days, a rate of regeneration which appears far more rapid than in most of the experiments reported by the other workers (2, 30–32). The level of protein fed in the synthetic ration is considerably higher than that present in 35 to 50 cc. of milk. The quality of protein and other constituents is also different. One may inquire as to the possibility that, after all, organic factors besides the inorganic may also be involved in the production of hemoglobin in the nutritional anemia of the rat on a whole milk diet. This phase of the problem is now being studied. Most of the recent work (2, 29–32, 41–43) in the field of experimental anemia has stressed the importance of

[2] This phase of the work was kindly carried out by Dr. George M. Robson of the Department of Pathology.

inorganic factors in hemoglobin formation. It is of interest, however, to note that the experiments of Sperry, Elden, Robscheit-Robbins, and Whipple (44) point to the possibility that in secondary anemia in the dog, the value of liver depends upon both inorganic and organic factors.

SUMMARY AND CONCLUSIONS.

Synthetic diets of the Karr-Cowgill type are adequate in respect to hemoglobin maintenance over long periods of time.

Low copper synthetic maintenance diets have been developed and have been found entirely suitable in rat feeding experiments. This type of diet has permitted a more exact evaluation of the significance of copper in hemoglobin production.

Rats which have become severely anemic upon a diet of whole milk, showed a prompt and striking recovery of hemoglobin when changed to synthetic rations, lower in copper (on the basis of the quantity fed per day) than the milk. One of these hemoglobin regenerative diets was an incomplete ration in that no vitamins had been added to it and the salt ration used contained only sodium chloride and an iron salt. The latter furnished the minimal level of 0.2 mg. of iron per rat per day. The same iron salt added in the same quantity to milk did not stem the progressive anemia. Without the addition of the iron, however, the synthetic ration was inadequate for hemoglobin production under the conditions of these experiments.

Some evidence was also presented which indicates that rats which have had copper in their ration are no more resistant to the onset of milk anemia than rats which have been on a low copper diet. This may have some bearing on the possibility that the effects of copper described by the Wisconsin investigators are not due to systemic action of the metal.

On the basis of the above experiments the specificity of copper in hemoglobin formation becomes questionable.

Both the Biazzo and the potassium ethyl xanthate methods for the determination of copper have been found inaccurate in certain respects and have been modified. In the controversy as to the unique importance of copper in hemoglobin synthesis, minute amounts of copper are of such moment that the method of determination becomes of utmost importance.

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ON THE MECHANISM OF PHLORHIZIN DIABETES.

II. THE RELATIONSHIP BETWEEN THE NUTRITIONAL STATE AND THE GLUCOSE TOLERANCE.*

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Although the early investigations upon the mode of action of phlorhizin (1, 2) pointed to the fact that its effect was essentially renal in character, some of the more recent work, chiefly that of Nash and Benedict (3), has been interpreted to indicate that an inability to oxidize carbohydrate occurs concomitantly with and independently of its kidney action.

In an earlier paper, Deuel, Wilson, and Milhorat (4) reported experiments which seemed to support the theory that the kidney was the sole site of the action of phlorhizin. It was suggested that the other conditions found in phlorhizinized fasted dogs which are normally ascribed to a true diabetes, such as the decreased glucose tolerance, the ketosis, and the elevated protein metabolism, might be traced to the carbohydrate starvation resulting from the primary kidney involvement. In support of this statement experiments were presented which showed no abnormalities in regard to carbohydrate metabolism when nephrectomized dogs were phlorhizinized. The protein metabolism, as judged by the rate of increase of nonprotein nitrogen in the blood, was not augmented by the administration of phlorhizin. The blood sugar levels did not progressively increase in the animals without kidneys on the administration of phlorhizin as one should expect if the power to oxidize carbohydrate were lost. Likewise, the respiratory quotients obtained with the nephrectomized animals which had been fasted were comparable with those obtained on normal fasting animals rather than on diabetic animals. The administration of carbohydrate to the phlorhizinized nephrectomized dogs was followed by the usual rise in respiratory quotient which one might expect if carbohydrate were being utilized. In phlorhizinized dogs having the renal function intact, a rise in the respiratory quotient occurred after 16 gm. of glucose in spite of the fact that a majority of the ingested sugar could be accounted for in that excreted.

Nash (5) has recently published additional experiments carried out on dogs rendered anuric by ligation of the ureters. Normal blood sugar values were maintained in phlorhizinized dogs after such a procedure as were obtained in the controls. Glucose tolerance curves on phlorhizinized dogs with ligated ureters simulated those obtained on non-phlorhizinized control animals although slightly higher results were found in the case of the former. The ketosis present in normal phlorhizinized dogs was abolished in each case by ureteral ligation. The rate of increase in the non-protein nitrogen of the blood proceeded at a uniform rate after the tieing off of the ureters. No significant differences in glycogen formation in the livers or muscles were noted in the phlorhizinized anuric animals from the control animals. These results essentially confirm those of Deuel, Wilson, and Milhorat although Nash differs in the interpretation of minor points. Nash believes that these results do not absolutely prove that the site of action of phlorhizin is entirely confined to the kidney.

Shorr, Loebel, and Richardson (6) using the Warburg technique for the determination of the respiratory metabolism of excised renal and testicular tissue from phlorhizinized rats, have also failed to demonstrate a difference in carbohydrate metabolism from that of the normal. The tissues obtained from phlorhizinized animals responded to the presence of glucose in the medium by an increase in oxygen consumption and a rise in respiratory quotient. These changes occurred even if an excess of phlorhizin were present in the nutrient medium. These investigators interpret their results as opposed to the theory that phlorhizin directly interferes with the oxidation of carbohydrate by the cells.

The present investigation was undertaken to obtain definite information about the relationship between carbohydrate ingestion of phlorhizinized dogs and their glucose tolerance. Although it seemed probable, as suggested in the earlier paper, that the diabetic symptoms obtained in phlorhizinized fasted animals are due to a secondary involvement brought on by the carbohydrate depletion of the tissues, it is highly desirable to substantiate this with definite experimental evidence. These data are compared with the experiments on the same dogs without phlorhizin at a later period when fasting or after the administration of carbohydrate the night previous to the test.

General Procedure.

In the glucose tolerance tests blood samples for glucose determinations were withdrawn approximately 30, 60, 90, 120, and 180 minutes after the administration of 16 gm. of glucose by stomach tube. In some cases where the blood sugar values had not returned to the original level, samples were taken at 4 and 5 hours.

The experiments were carried out on female dogs. The phlorhizin was administered with the same technique as that previously employed (4) except that phlorhizin was administered in oil only. The glucose tolerance tests on the fasting phlorhizinized animals were usually made on the 5th day of phlorhizin and the 7th day of fasting. The experiments made on the well fed phlorhizinized animals were generally carried out on the day following the control experiment in the fasting state. For increasing the carbohydrate content in the tissues of the previously fasted and phlorhizinized animals, glucose was given in two experiments by stomach tube at constant rate for 6 to 8 hours previous to the tolerance test. In most experiments, however, the improved nutritional condition as regards carbohydrate was brought about by the administration of a large dose of sucrose on the night previous to the tolerance test. In one case a large amount of bread was fed.

Nitrogen estimations on the urine were made by the Kjeldahl procedure; urinary sugar was determined by the Bertrand method; urine and blood acetone determinations were carried out by the technique of Van Slyke (7). To find the blood sugar value the procedure of Shaffer and Hartmann was used. In some cases when it was desired to ascertain the extent of the sucrosuria, the determinations of reduction were made on the urine before and after hydrolysis. This latter process was performed by boiling an aliquot of the urine with sulfuric acid for 1 minute (10 drops of concentrated H_2SO_4 to 25 cc. of urine), followed by neutralization and making up to volume.

Blood was usually drawn from the jugular vein although in some instances it was obtained from the saphenous or median veins. Urine separations were by catheter.

Experimental Results.

1. Experiments on Phlorhizinized Animals.—One experiment was carried out on Dog 5 in which the glucose tolerance curve was determined after the administration of 10 gm. doses of glucose 3 hours apart. The first response represented that which occurs in a fasted phlorhizinized animal with a considerable carbohydrate depletion in the tissues. On the other hand the second result is that given by an animal with the blood sugar at a somewhat higher level, with the ketosis abolished, and with the carbohydrate at a

somewhat higher level, than in the first experiment. After the administration of 16 gm. of glucose, less than 50 per cent has

TABLE I.

Glucose Tolerance Curves after Administration of 10 Gm. of Glucose at 3 Hour Intervals to a Normal and Phlorhizinized Fasting Dog (Dog 5).

Values are measured in mg

Before glucose.	Time after glucose administration, min.				Remarks.
	45	75	120	180	
100	186	135	102	96	Dog fasted 2 days.
96	206	119	87	91	3 hrs. after previous experiment.
53	108	137	120	90	5th day of phlorhizin and fasting.
90	131	140	106	73	3 hrs. after previous experiment.

TABLE II.

Blood Sugar before and after Administration of 16 Gm. of Glucose during Constant Glucose Injection in Phlorhizinized Dogs.

Values are measured in mg.

Before glucose.	Time after glucose administration, min.						
	30	60	90	120	180	240	300

Dog 8.   7.5 gm. glucose in 300 cc. H_2O hourly intraperitoneally for 6 hrs. previous.   Dog unanesthetized.   D:N ratio at start was 27.05:1.

218*	205	272	292	293	213	202	

Dog 10.   4 gm. glucose by stomach tube at constant rate hourly in 200 cc. for 7 hrs. previous.   Dog unanesthetized.   D:N ratio at start was 15.89:1.

141†	153	165	170	162	159	154	122

* Previous blood sugar values preceding 16 gm. dose of glucose were 209, 246, and 225.

† Previous blood sugar values preceding 16 gm. dose of glucose were 146, 155, 126, 138, and 132.

been excreted within a 3 hour period (8). These data are given in Table I.

In the first experiment after phlorhizin the maximum rise is 82 mg. and the blood sugar is considerably elevated at 180 minutes.

TABLE III.

Blood Sugar Value of Phlorhizinized Dogs before and after 16 Gm. of Glucose in Fasting and after Previous Carbohydrate Feeding.

Values are measured in mg.

Experiment No.	Nutritional condition.	D:N ratio.		Blood acetone	Before glucose	Blood sugar. Min. after glucose administration.								
		Before.	After.			30	60	90	120	180	240	300	360	480
				Dog 5; weight, 12.8 kilos.										
I	P 5d.; F 6d.	3.42	9.90	27.5		76	116	136	152	153	116	88	80	
II	" 6d.; 250 gm. CH 14 hrs. before.	20.45	26.96	0.0		113	152	183	152	112				
III	P 7d.; 200 gm. CH 14 hrs. before.	17.01	20.50			114	143	135	144	104				105
				Dog 8; weight, 12.4 kilos.										
I	P 5d.; F 10d.	3.73	12.72	21.0		68	127	185	228	221	137	105		
II	" 6d.; " 6d.	3.62	7.56	9.0		79	141	200	200	235	168	108	97	
III	" 7d.; 200 gm. CH 13 hrs. before.	4.76	8.43			110	129	145	147	136	125			
				Dog 10; weight, 7.5 kilos.										
I	P 5d.; F 5d.	4.30		1.5		88	160	180	193	192	135	95	78	
II	" 8d.; " 2d.	3.55	13.78	2.5		80	110	145	167	175	125	87		
III	" 6d.; " 7d.	3.58	11.27	24.1		67	122	164	191	217	203	136	94	85
IV	" 7d.; 150 gm. CH 15 hrs. before.	17.57		0.0		96	186	202	180	116	88			
V	P 8d.; 150 gm. CH 14 hrs. before.	23.39		0.0		103	141	192		122	85			
				Dog 11; weight, 14.3 kilos.										
I	P 7d.; F 1d.*	3.50		38.1		70	130	167	183	158	140	109		
II	" 8d.; 375 gm. CH 16 hrs. before.†	25.05		1.0		153	155	162	163	152				

Time after glucose is approximate.   P is phlorhizin; F, fasting; CH, carbohydrate; d., days.

* CO_2-combining power of blood is 27.0 volumes per cent.

† CO_2-combining power is 42.0 volumes per cent.

In the second experiment the increase is only 50 mg. and the blood sugar is appreciably reduced from the initial level at 3 hours.

The glucose tolerance curves were also made on two phlorhizinized fasting dogs (Dogs 8 and 10) and compared with the results when the carbohydrate depletion of the tissues had been abolished by a constant introduction of glucose solution intraperitoneally or by stomach tube for 6 to 8 hours previous to the administration of an additional 16 gm. of glucose for the tolerance test. The constant introduction of glucose continued until the end of the experiment. The data on these experiments are given in Table II. The most satisfactory demonstration of the differences in carbohydrate metabolism between the carbohydrate-depleted and carbohydrate-fed animals is shown in the six experiments on four dogs given in Table III. In each case a large amount of sucrose or starch (bread) was given on the night preceding the tolerance test. Since these experiments were carried out on the same dogs as those reported in Table II, the glucose tolerance tests of the fasting phlorhizinized dogs are included only in Table III.

In the experiments reported in Table II, the maximum rise in blood sugar with Dog 8 was only 75 mg. and the blood sugar had returned to the initial level at 3 hours. On the other hand when this same animal was fasted and phlorhizinized, the blood glucose reached a value 160 mg. higher than the starting value and was still above the initial level after 5 hours (Table III). With Dog 10 the greatest increase noted amounted to 29 mg. when the glucose test meal was superimposed on the constant glucose administration. This same animal in the carbohydrate-depleted state showed a rise in blood sugar of 105 and 95 mg. and required 5 hours for the glycemia to return to the original value.

The glucose tolerance was uniformly much higher in all of the six experiments on the phlorhizinized dogs which had received a heavy carbohydrate meal on the previous night (Table III) than that obtained on the same animals when in a state of carbohydrate depletion. This improved condition is reflected in the shorter period of postprandial hyperglycemia as well as the slighter rise from the pre-glucose level. In three experiments the increase in blood sugar lasted less than 2 hours while in two other experiments values well below the preprandial level were found after 3 hours.

In only one case (Dog 8) a slightly elevated result was obtained after 3 hours although the increase is small and the maximum rise of 37 mg. does not compare with the values on the fasting phlorhizinized animals. The discrepancy in the case of this animal is probably due to only a partial retention of the administered carbohydrate (probably because of vomiting the bulk of it). That such is the case is indicated by the relatively low D:N ratio in contrast with the values obtained on the other animals under a like dietary régime.

After the large doses of carbohydrate the blood sugar was considerably elevated above the usual level for fasting phlorhizinized dogs on the following morning. The blood sugar was stabilized at this higher level 14 hours after the administration of excessive amounts of carbohydrate and would have remained at approximately this value had glucose not been fed. This is indicated by the regularity in results obtained after feeding the glucose test meal. Also with Dog 5, it was demonstrated that the blood sugar remained at about the pre-glucose level from the 3rd to the 6th hour after the administration of this sugar. Sucrosuria which occurred in some cases when very large amounts of this sugar were given, was always absent from the urine obtained immediately before the tolerance experiment. In all cases the acetonemia which had existed prior to the carbohydrate feeding was abolished or greatly decreased. Acetonuria was likewise abolished as was first shown by Wierzuchowski (9).

On the other hand in the seven experiments on the same animals when in a state of carbohydrate depletion the blood sugar value had in no case returned to the initial level after 4 hours nor was such the case in three of four experiments after five hours.

A high excretion of glucose resulted in the urine of the animals fed large quantities of carbohydrate demonstrating that the kidney was still functioning in a typical manner toward phlorhizin despite the normal blood sugar, the abolition of the acidosis, and the normal tolerance curves obtained. The glucose and nitrogen excretion in the urine was determined daily from the 2nd or 3rd day following the first administration of phlorhizin. However, only those values of direct importance in interpreting the data in Table III are included in Table IV.

There is a marked decrease in protein metabolism in each case

TABLE IV.

Urine Analyses on Phlorhizinized Dogs before and after Receiving Carbohydrate.

Dog No.	Days after phlorhizin.	Urine sugar. Per period.	Hourly.	Urine N. Per period.	Hourly.	D:N ratio.	Sucrose in urine as glucose.	Acetonuria.	Diet.
		gm.	gm.	gm.	gm.		gm.	mg. hourly	
5	5	24.30		6.81		3.57			Fasted 6 days.
		15.37	1.77*	4.50	0.505*	3.42		72.8	
	5	20.13	4.94	2.03	0.500	9.90		19.8	16 gm. glucose (I).
	6	28.04		2.67		10.50	36.38		250 gm. sucrose.
		42.34		2.27		18.68	27.06		
		5.38	4.26	0.26	0.288	20.45	0.0	0.0	
	6	14.26	4.26	0.53	0.158	26.96		0.0	16 gm. glucose (II).
	7	52.60		2.37		22.23	9.80	0.0	200 gm. sucrose.
		11.50	2.97	0.68	0.141	17.01	1.71	0.0	
	7	11.17	3.55	0.54	0.159	20.50	0.0	0.0	16 gm. glucose (III).
8	5	23.56	0.99	6.31	0.265	3.73		68.3	Fasted 10 days.
	5	16.35	3.54	1.28	0.278	12.72		14.0	16 gm. glucose (I).
	6	13.81		4.01		3.42			Fasted 6 days.
		3.15	0.85	0.87	0.286	3.62		12.9	
	6	11.30	5.39	1.49	0.710?	7.56		0.0	16 gm. glucose (II).
	8	23.16		3.32		6.98	160.78†	0.0	200 gm. sucrose.
		1.95	1.07	0.41	0.157	4.76	0.0	0.0	
	8	5.56	1.72	0.66	0.204	8.43		0.0	16 gm. glucose (III).
10	5	35.95		5.29		6.67			Urine lost for I.
		4.18	2.12	0.97	0.320	4.30		30.9	
	8	43.72	1.71	12.31	0.483	3.55		18.9	
	8	19.21	4.61	1.41	0.339	13.58		0.0	16 gm. glucose (II).
	6	17.49	1.17	4.88	0.327	3.58		93.3	Fasted 7 days.

* In this case as well as in the following data in this table, separate analyses have been made on the catheter specimen. In such instances the hourly glucose and nitrogen are computed on the total period including the catheter sample. In this table the hourly average of these constituents is opposite the last sample (catheter) taken in any particular period but is based on the total excretion for the whole period.

† This was probably vomited. This would acount for comparatively low urinary glucose and the low D:N ratio. Since 200 gm. of sucrose were given and only 160 gm. excreted, some was retained.

TABLE IV—*Concluded.*

Dog No.	Days after phlorhizin.	Urine sugar.		Urine N.		D:N ratio.	Sucrose in urine as glucose.	Acetonuria.	Diet.
		Per period.	Hourly.	Per period.	Hourly.				
		gm.	gm.	gm.	gm.		gm.	mg. hourly	
10	6	18.26	3.29	1.62	0.290	11.27		23.0	16 gm. glucose (III).
	7	56.04		3.13		17.87	45.11		150 gm. sucrose.
		2.11	2.95	0.18	0.168	11.47	0.0	0.0	16 gm. glucose (IV). Urine lost.
	8	44.06		1.91		23.07	68.94		150 gm. sucrose.
		0.99	2.05	0.06	0.090	16.98	0.0		16 gm. glucose (V). Urine lost.
11	7	8.07		1.99		4.05			Fasted 1 day. Lactose (16 gm.) on each of 2 previous days. Fasted 5 days before.
		6.01	1.16	1.72	0.307	3.50			
	7								16 gm. glucose (I).
	8	107.30		4.36		24.57			375 gm. CH.
		5.31	4.79	0.21	0.194	25.05			16 gm. glucose (II) following this period. Urine lost.

following the ingestion of large amounts of carbohydrate food. This occurs concomitantly with the abolition of the acidosis. The fall in protein metabolism which occurs after the ingestion of 16 gm. of glucose as noted by Deuel and Chambers (8) does not reach its maximum until about the 6th hour. For that reason, the decrease is not marked in the samples collected 3 hours after the feeding of glucose as was the practice in the present experiments. After the administration of the excessive dose of sucrose, an average of 3 to 5 gm. of glucose was excreted hourly for an extended period, demonstrating the fact that the kidney still reacted to phlorhizin in a normal manner despite the elimination of the other diabetic symptoms.

2. Experiments on Normal Dogs.—The glucose tolerance of

three of the four animals used in the phlorhizin experiments was determined after the glycosuric action of phlorhizin had worn off and the nutritional condition of the animal had returned to a normal one.   Glucose was fed and the blood sugar followed in each case after a 3 or 4 day fast as well as after a period of heavy carbohydrate feeding.   The results are tabulated in Table V.

TABLE V.

Blood Sugar Values of Normal Dogs before and after 16 Gm. of Glucose in Fasting and after Previous Carbohydrate Feeding.

Values are measured in mg.

Dog No.	Experiment No.	Nutritional condition.	Blood sugar.						
			Before glucose.	Time after glucose administration, min.					
				30	60	90	120	180	240
5	IV	Fasting 1 day.*	100	·	186†	135‡	102	96	
	V	3 hrs. after Experiment IV.*	96		206†	119‡	87	91	
	VI	Fasting 6 days.	122	195	206	225	174	130	124
	VII	150 gm. sucrose 22 hrs. before.	112	150	142	108	101	108	
8	IV	Fasting 3 days.	98	183	207†	207‡	211	141	
	V	" after phlorhizin.	108	154	208	252	223	100	
	VI	" 4 days.	93	172	194	150	120	90	
	VII	150 gm. sucrose 14 hrs. before.	112	144	123	122	106	109	
10	VI	Fasting 4 days after Experiment VII.	110	168	208	186	109		
	VII	150 gm. sucrose 14 hrs. before.	111	146	153	105	87		

* 10 gm. glucose only.
† 45 minutes after.
‡ 75 minutes after.

In general the glucose tolerance is considerably decreased by a short period of fasting.   This is demonstrated by a greater rise in blood sugar and a more prolonged hyperglycemia after the glucose meal.   With Dog 8 after 3 days of fasting a maximum increase of 113 mg. occurred in the blood sugar after the ingestion of 16 gm. of glucose and the pre-glucose level had not been reached after 3

hours. On the other hand, when 150 gm. of sucrose had been fed on the previous night following a heavy carbohydrate diet on the preceding days, the administration of 16 gm. of glucose caused a maximum rise of only 32 mg. in the blood sugar level. It had fallen below the initial value within 2 hours. Similar differences were noted in the experiments obtained on the other two animals.

<div align="center">DISCUSSION.</div>

The experiments here reported offer additional proof that no intrinsic impairment of the ability to oxidize carbohydrate exists in phlorhizinized animals. When this drug acts on the kidney, the available glucose is mobilized and drained. Hence carbohydrate ceases to be oxidized on account of the low concentration in the blood and tissues. Under these conditions the stimulation of insulin production does not occur. In the absence of the metabolism of carbohydrate, acidosis ensues and the glucose tolerance becomes lowered.[1]

On the subsequent administration of a small amount of sugar a diabetic glucose tolerance curve is obtained and the carbohydrate is almost quantitatively eliminated as extra sugar in the urine. One might interpret these findings as indicative of a decrease in the ability to utilize carbohydrate which might be caused by the direct union of phlorhizin with the carbohydrate receptors of the cells. However, when large amounts of sucrose are fed, the presence of phlorhizin does not significantly alter the normal carbohydrate metabolism of the animal. The ketosis disappears, the postprandial hyperglycemia becomes much less pronounced, and the carbohydrate oxidation proceeds at a normal rate. Glycosuria persists which would indicate that the action of this drug on the kidney was still maintained. The rate of elimination of glucose in the urine was as high as 4.5 gm. hourly for as long as 14 hours after the sucrose or carbohydrate feeding. This would tend to prove that the kidneys were phlorhizinized despite the excess of carbohydrate present in the animals. In the case of Dog 11, this amounts to an increase of more than 300 per cent in the excretion of glucose by the kidney.

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[1] The decreased tolerance is probably not the result of acidosis as shown in the following paper.

The most likely explanation of the action of phlorhizin in producing symptoms simulating the diabetic in fasted dogs is that such a condition corresponds to the starvation ketosis in which decreases in glucose tolerance are also noted. The same qualitative differences between the glucose tolerance curves obtained on fasting and carbohydrate-fed phlorhizinized dogs were found in the same animals when subjected to like alterations in the dietary régime in the non-phlorhizinized state. Such decreases in glucose tolerance have also been noted in simple fasting by the conclusive experiments of du Vigneaud and Karr (10) as well as by Harding and van Nostrand (11). Chambers[2] has demonstrated that when 50 gm. of glucose are administered to a normal animal which has fasted for a prolonged period, a very high blood sugar occurs 4 hours after the feeding, a considerable glycosuria is present, and that no rise in respiratory quotient follows. When the same amount of glucose was administered daily for 4 days thereafter, the hyperglycemia became progressively lower, glycosuria disappeared, and higher and higher R. Q.'s were obtained, indicating a successive increase in the carbohydrate oxidation. Tolstoi (12) has found similar differences in the glucose tolerance after a prolonged protein-fat diet.

Chart I shows the blood sugar values in Dog 5 after the feeding of 16 gm. of glucose in the phlorhizinized and non-phlorhizinized state when fasting and after high carbohydrate feeding.

It is not necessary to assume any combination with the carbohydrate receptors of the cells in order to explain the diabetic glucose tolerance curves found in such a physiological condition as fasting, or to account for the inability to oxidize carbohydrate or even the glycosuria which may occur under such a régime. There seems no greater necessity for postulating such a union between phlorhizin and the carbohydrate receptors of the cells, as Nash and Benedict have done, in order to elucidate the post-glucose hyperglycemia, the diabetic glucose tolerance curves, and the inability to oxidize carbohydrate which occurs in these animals when carbohydrate is first given after a period of fasting coupled with the injection of phlorhizin. Normal responses are obtained in

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[2] Chambers, W. H., presented at the Federation of American Societies for Experimental Biology in Chicago, March, 1930.
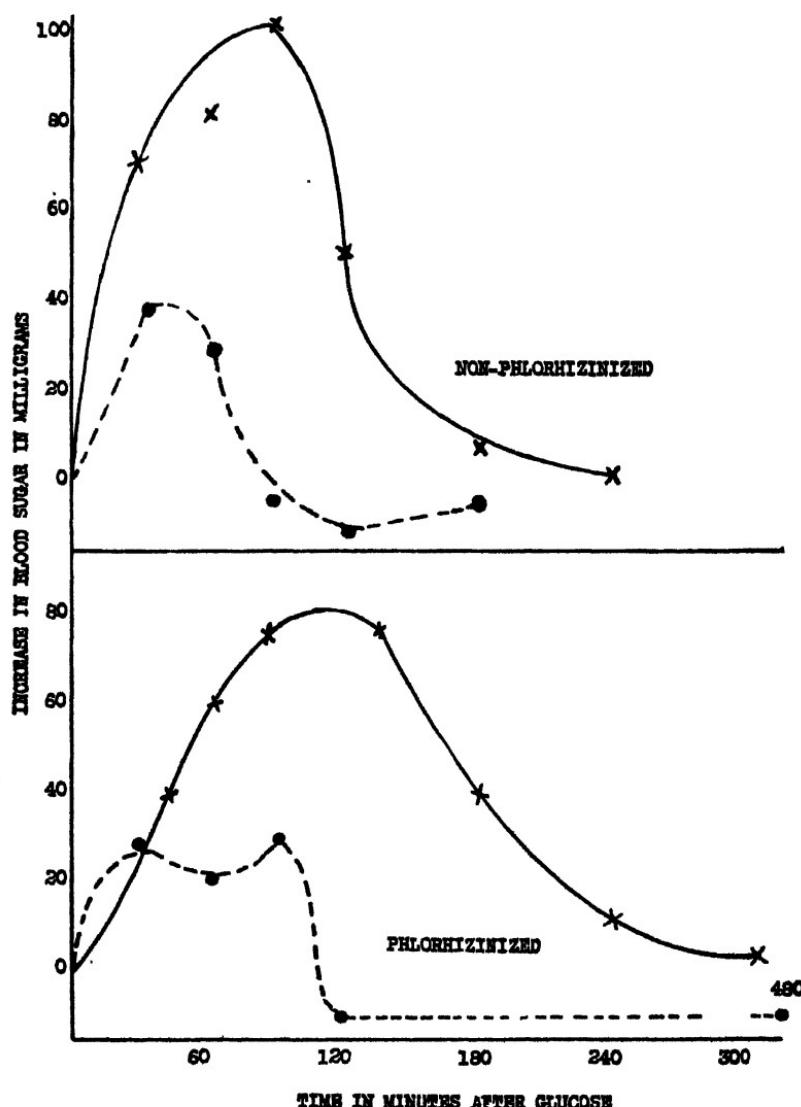
CHART I. Glucose tolerance curves on Dog 5 in the phlorhizinized and non-phlorhizinized state. The solid lines in each case represent the blood sugar values after 16 gm. of glucose were fed to the animal previously fasting. Each individual blood sugar determination is indicated by a cross. The broken lines show the results in this test on the same dog when the animal possessed large stores of carbohydrate. Individual determinations in this case are marked by large dots.

either the normal or the phlorhizinized animal after several administrations of carbohydrate.

Nash (5) has called attention to the fact that an increase in protein metabolism was reported in our earlier work before any fall in blood sugar was noted. It now seems problematical as to whether this early increase is to be traced to a depletion of glycogen reserves in the tissues as Nash suggests or to a decreased threshold for urea. McKay[3] has found that in addition to the decrease in the glucose threshold after phlorhizin there is also a lowering in that for chlorides. It seems possible that less urea might be likewise reabsorbed under such conditions and so account for the increased nitrogen elimination.

SUMMARY.

When a standard glucose meal was administered to fasting phlorhizinized dogs, the glucose tolerance curves obtained were distinctly of the diabetic type. When the same amount of this sugar was given to the identical animals about 14 hours after the administration of a large dose of carbohydrate, the glucose tolerance was much increased as indicated by an early return of the blood sugar to the postprandial level and by the much smaller rise obtained above the initial value. The same qualitative variations in response were noted in the same animals without phlorhizin when fasted as compared with that obtained 14 hours after heavy carbohydrate feeding. These results are offered as additional proof that the action of phlorhizin is primarily renal in character and that no intrinsic impairment in the ability to oxidize carbohydrate exists under such conditions.

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THE RELATION BETWEEN ALKALI DEFICIT AND GLUCOSE TOLERANCE IN THE DOG.

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A direct relationship seems to exist between the frequency of stimulation of the islands of Langerhans by carbohydrate ingestion and the amount of insulin which is produced on a subsequent excitation in this way. In the previous paper (1) it was shown that the glucose tolerance was reduced in normal or phlorhizinized dogs during fasting when a single glucose meal of 16 gm. of this sugar was fed. On the other hand, when an identical amount of this carbohydrate was given to the same animals in either condition but with the nutritive state considerably improved by the administration of much carbohydrate 14 to 16 hours previously, a marked decrease in the height of the blood sugar rise and a greatly shortened period of postprandial hyperglycemia resulted.

In practically all of the cases studied previously, in which the rate of disappearance of glucose from the blood was retarded, ketosis was present as a complicating factor. On the administration of carbohydrate an improvement in glucose tolerance occurred concomitantly with the abolition of the acidosis. Such is true with the decreased tolerance noted in fasting, with the diabetic in whom the glucose tolerance improves when insulin is injected—a condition which at the same time clears up the ketosis—and also in the case of phlorhizin glycosuria as shown by us in the previous paper (1).

The presence of an acidosis has frequently been interpreted as the cause of the decreased tolerance noted. Adrenalin, by producing vasoconstriction, results in a temporary asphyxia, lactic acid is formed in increased amounts, and glycogenolysis therefore occurs. Asphyxial glycosuria and that following anesthesia may

be so explained.   Du Vigneaud and Karr (2) have found that the decreased tolerance which is present in fasting may be prevented by the administration of alkali although these investigators believe that acidosis is not the predominant factor.

TABLE I.

Glucose Tolerance Curves of Dogs on High Carbohydrate Diet with Alkali Deficit.

Blood sugar values are measured in mg. and CO_2-combining power in volumes per cent.

CO_2-combining power.		Blood sugar.							Remarks.
Before NH_4Cl	After NH_4Cl	Before glucose.	Min. after glucose administration.						
			30	60	90	120	180		
Dog 5; weight, 17.5 kilos.									
55.3	33.9	119	150	140	147	118			15 gm. NH_4Cl by stomach tube 15 hrs. before test.
	34.2	116	163	153	145	147	112		10 gm. NH_4Cl given but largely vomited. Sucrose given also vomited.
Dog 8; weight, 15.5 kilos.									
50.7	25.3	120	143	143	124	122			10 gm. NH_4Cl given with 500 gm. bread 17 hrs. before test. No vomiting.
	25.9	108	125	124	118	117			5 gm. additional NH_4Cl given with bread. Day following previous experiment.
Dog 10; weight, 11.4 kilos.									
49.7	16.9	130	140	134	131	128			10 gm. NH_4Cl subcutaneously 16 hrs. before test.
	28.2	128	159	147	143	143	125		Following day. No additional NH_4Cl. About 80 gm. sucrose given 16 hrs. before test.

The present investigation was carried out to compare the rate of the removal of glucose from the blood in a well fed dog having a decreased alkaline reserve with that of the same animal under a

similar dietary régime but having a normal alkaline reserve. Since it is impossible to bring about such an alkali deficit by a ketosis and at the same time have abundant carbohydrate stores, this condition was produced by the administration of NH_4Cl in 10 to 15 gm. doses according to the method of Haldane (3). The extent of the effect was determined by the estimation of the CO_2-combining power of the plasma according to the procedure of Van Slyke (4). The other technique for the tolerance tests was similar to that in the previous paper. Table I shows the results of the six tests on three dogs. All of these animals were used in the earlier work to which reference should be made for comparison of the tolerance curves with those of the normal animal on the same dietary régime.

The results in all cases show as high a tolerance as was noted in the same animals when normal and on a high carbohydrate diet. (See the previous paper for comparison.) The maximum rise in blood sugar was 47 mg. above the initial level. In most cases the blood sugar was at the pre-glucose level within 2 hours and in all cases within 3 hours after the test meal was given. A severe decrease in alkaline reserve was brought about by the NH_4Cl, the normal CO_2-combining power of 55 to 50 volumes per cent being lowered to 34, 25, and in one case to 16 volumes per cent.

One must conclude that a compensated acidosis in itself is not a factor which prevents the ready removal of glucose from the blood. This proceeds in a perfectly satisfactory manner if sufficient carbohydrate has been fed 14 to 18 hours before the glucose test is given.

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p-AMINOPHENYLGUANIDINE HYDROIODIDE.

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INTRODUCTION.

The discovery by Frank (1,2) and his coworkers of synthalin, a polymethylene diguanidine, with its marked hypoglycemic action, has stimulated research on synthetic guanidine types. The Rathke (3) synthesis has been sucessfully applied to the preparation of various substituted guanidines and diguanidines by Phillips and Clarke (4), Schering-Kahlbaum Company (5), Bischoff (6), Kumagai, Kawai, Shikinami, and Hosono (7), Smith (8), and Heyn (9). In general a suitable amine or diamine has been condensed with S-methylisothiourea sulfate, the latter usually having been prepared by the method of Arndt (10). The condensation also has been carried out with satisfactory results with an amine or a diamine and S-methylisothiourea hydroiodide.

If an amine is used a substituted monoguanidine results, whereas if a diamine is used a diguanidine usually is produced. An apparent exception recently has been observed in the case of p-phenylenediamine, which, when condensed with S-methylisothiourea hydroiodide, gives a substituted monoguanidine, namely, p-aminophenylguanidine as the hydroiodide salt, and not a diguanidine as might be expected. Even when the S-methylisothiourea hydroiodide has been used in excess of the molecular ratio of 2 mols to 1 mol of diamine, the monoguanidine always has resulted. The probable mechanism of the condensation, as evidenced both by the evolution of methyl mercaptan and by the structure of the reaction product, can be illustrated by the following equation:

Although *p*-aminophenylguanidine and its salts have not been found recorded in the literature, its isomers, phenylaminoguanidine and α-amino-α-phenylguanidine have been described by Pellizzari (11), and its methylated derivative, *p*-guanidinodimethylaniline, has been prepared by Bischoff (6). The relationships between these compounds can be seen from their structural formulas.

p-Aminophenyl- guanidine hydroiodide.	Phenylamino- guanidine.	α-Amino-α-phenyl- guanidine.	*p*-Guanidino- dimethylaniline.
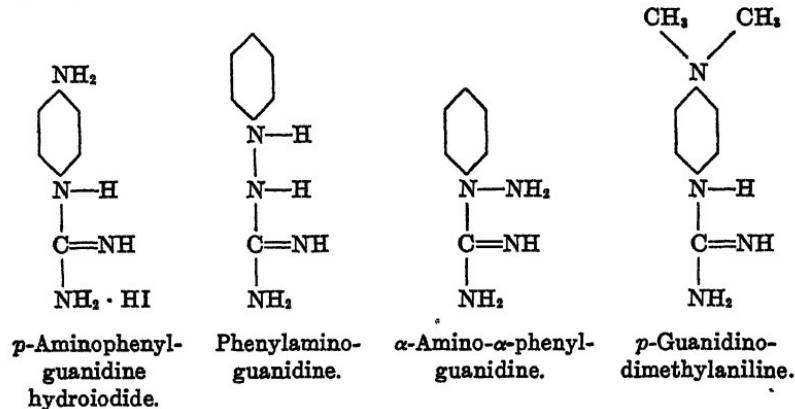
Several aromatic guanidines which have been examined recently for hypoglycemic action by Cannavo (12) and by Bischoff, Sahyun, and Long (13) have proved to be very toxic and produced practically no hypoglycemia. A possible exception was found in diguanylbenzidine, prepared in impure form by Bischoff (6, 13), and reported as having a physiological action similar to that of synthalin.

Despite the unsatisfactory results obtained with aromatic guanidines in previous investigations, the similarity of structure between agmatine (1), 1-guanidino, 4-amino-butane, which has been found to produce hypoglycemia, and *p*-aminophenylguanidine hydroiodide suggested the idea of examining the latter compound

for hypoglycemic action. In a preliminary physiological investigation p-aminophenylguanidine hydroiodide produced a marked hypoglycemia without apparent toxic symptoms.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*I. Preparation of p-Aminophenylguanidine Hydroiodide.*</div>

<div align="center">*Materials.*</div>

Thiourea.—The thiourea used was a commercial product of high quality, supplied by the American Cyanamid Company. It melted at 175–177°.

Methyl Iodide.—This was prepared in the usual way by reacting absolute methyl alcohol with resublimed iodine and red phosphorus. The product boiled at 43°.

S-Methylisothiourea Hydroiodide.—This substance was synthesized by the reaction of methyl iodide with thiourea, the method of Wheeler and Merriam (14) being followed, in general. Since the directions as reported by these authors are extremely meager, it was found necessary to work out the experimental procedure. A method was developed which gave such satisfactory results that it is considered worth reporting at this time. In detail, the synthesis is as follows:

38 gm. (½ mol) of thiourea, very finely pulverized, were placed in a 500 cc. round bottom flask. The thiourea was then moistened with about 15 cc. of 95 per cent alcohol. A long reflux condenser was then attached to the flask, which was so arranged that it could easily be cooled if necessary. Through the condenser, in a single addition, were added 76 gm. (½ mol plus 5 gm. excess) of methyl iodide. After several minutes the reaction began with a considerable evolution of heat. Due to the high volatility of the methyl iodide, it seemed advisable at this stage to cool the reaction vessel in order to prevent loss of the halide. This was accomplished by immersing the flask in a bath of ice water. When the reaction subsided a yellow solution resulted, which, upon cooling and standing for ½ to 1 hour, solidified almost completely to a cream-colored mass. The solid was broken up, 100 cc. of absolute ether added, and the thick mush filtered with suction. It was washed on the funnel with 3 100 cc. volumes of absolute ether. After drying in the air, the product was a white crystalline solid,

melting at 116–117°, which is in good agreement with the melting point recorded in the literature for S-methylisothiourea hydroiodide, namely, 117°. The yields obtained in several runs were as follows:

Thiourea used. gm.	Actual yield. gm.	Theoretical yield. gm.	Per cent yield.
38	108	109.0	99.1
38	109	109.0	100.0
38	102	109.0	93.6
76	207	218.0	95.0
57	162	163.5	99.1
76	208	218.0	95.4

p-Phenylenediamine.—This reagent was an Eastman product, melting at 137–139°.

Condensation of p-Phenylenediamine with S-Methylisothiourea Hydroiodide.

A. In Absolute Alcohol.—The first attempts to condense *p*-phenylenediamine with S-methylisothiourea hydroiodide were carried out in absolute ethyl alcohol. Believing that the two amino groups of the diamine would react, 2 mols of the isothiourea were used, to 1 mol of *p*-phenylenediamine. The reactants were heated on a boiling water bath for 5 days. At the end of this time, the hot reaction mass was a deep violet-colored solution. The liquid was evaporated under a vacuum. After a time a crystalline substance deposited. This was filtered off, washed once with cold 95 per cent alcohol, and dried in a vacuum over sulfuric acid. The dried crystals had a grayish white color and melted at 205–210°. This crude product was soluble in alcohol and water but insoluble in acetone. The substance, when bone-blacked and recrystallized from hot 95 per cent alcohol, produced shining white crystals which melted sharply at 209–210°. A sodium fusion and qualitative test for elements revealed the presence of nitrogen and iodine and the absence of sulfur. A quantitative analysis for nitrogen and iodine showed the following results.

Nitrogen (by Kjeldahl)............................ 20.01 per cent
Iodine (as AgI).................................... 45.44 " "

These data do not agree with those calculated for a diguanidine but are in very close agreement with the theoretical calculations

for the hydroiodide salt of a monoguanidine, namely, *p*-amino-phenylguanidine.

	Calculated for $H_2NC_6H_4N_3CH_4 \cdot HI$.	Found.
Nitrogen...........................	20.15	20.01
Iodine........ ;....................	45.65	45.44

If the crystalline product is the hydroiodide salt of *p*-amino-phenylguanidine as the analytical data indicate, then it should be possible to form the same compound by condensing *p*-phenyl-enediamine with S-methylisothiourea hydroiodide in equimolecular ratios, instead of using 2 mols of the isothiourea to 1 of the diamine. When such a condensation was attempted it produced the same compound as was obtained in the first reaction. The new reaction product melted at 209–210°, and also produced iodide ion in water solution. A mixed melting point determination gave further evidence to prove that the two compounds were identical. Subsequent experimentation showed that the time of reaction and also the volume of reaction medium could be greatly reduced without decreasing the quality of the product or the yield. The experimental details as finally developed, were as follows:

21.6 gm. (0.2 mol) of *p*-phenylenediamine and 43.6 gm. (0.2 mol) of S-methylisothiourea hydroiodide were placed in a 200 cc. round bottom flask fitted with a reflux condenser. 75 cc. of absolute ethyl alcohol were added through the condenser. The reaction vessel was placed in a boiling water bath and allowed to remain there for 2 hours. An almost immediate evolution of methyl mercaptan made it advisable to carry out the condensation under a hood. After 2 hours the flask and its contents were cooled either by running water or by immersion in an ice bath. Upon cooling, the reaction mass crystallized and became almost solid. The crystals were filtered off with suction, and were washed once on the funnel with cold acetone. This acetone washing removed most of the violet color, which seemed to form as the condensation took place. The material was bone-blacked and recrystallized once from hot water and once from hot 95 per cent alcohol. The final product was a white crystalline solid melting at 209–211°. The yields obtained were low, an average being 35 per cent.

B. In Water.—*p*-Aminophenylguanidine hydroiodide can be made also by condensing *p*-phenylenediamine with S-methyliso-

thiourea hydroiodide in water instead of in alcohol. When the reaction was carried out in an aqueous medium no advantages were gained from the points of view of time of reaction, quality of product obtained or ease of manipulation, but a decided increase in yield was observed. A typical water condensation consisted of the following experimental details.

21.6 gm. (0.2 mol) of *p*-phenylenediamine and 43.6 gm. (0.2 mol) of S-methylisothiourea hydroiodide were placed in a 200 cc. round bottom flask. To the reactants were added 75 cc. of water. The flask was then placed in a boiling water bath (under a hood) and allowed to remain there for 2 hours. Upon cooling in ice water a grayish crystalline material deposited in the flask. The reaction product was filtered off with suction, washed once with cold acetone, bone-blacked, and recrystallized once from hot 95 per cent alcohol and once from hot water. The final product was white and melted at 209–210°. The yield, calculated on the basis of the purified compound, was 54 per cent. Analyses for nitrogen and iodine on the crystalline material showed it to be *p*-aminophenylguanidine hydroiodide. The analytical data were as follows:

	Calculated for $H_2NC_6H_4N_3CH_4 \cdot HI$.	Found.
Nitrogen (by Kjeldahl)................	20.15	20.00
Iodine (as AgI)......................	45.65	45.47

II. General Characteristics and Solubility of p-Aminophenyl-guanidine Hydroiodide.

The purified product is pure white and very stable when dry. Its aqueous or alcoholic solutions become colored on standing, and especially if heated, probably due to oxidation. If slightly impure the compound may possess a faint tan color, and if very impure may even be brown. As might be expected, it produces iodide ion in aqueous solution, as evidenced by the precipitation of silver iodide upon the addition of silver nitrate.

In order to gain more than a mere qualitative knowledge of the solubility relationships of *p*-aminophenylguanidine hydroiodide, its solubility in various common solvents was quantitatively determined at 25°. The results were as follows:

Solvent.	Solubility in parts per 100.
Water..	6.9
Ethyl alcohol (absolute)..............................	2.9
Methyl " "	16.9
" acetate...	0.7
Acetone..	2.3
Ether (absolute).......................................	0.1
Benzene..	0.1
Chloroform..	0.0

Several attempts have been made to isolate the free base but all have proved unsuccessful to date.

The sulfate, chloride, and nitrate of p-aminophenylguanidine are being investigated at present but insufficient data prevent reporting upon them at this time.

III. Hypoglycemic Action of p-Aminophenylguanidine Hydroiodide.

Three series of experiments were carried out, with normal rabbits as experimental animals, none of which had ever been used in any previous experimentation. The rabbits were fasted for 24 hours prior to being used. The required amounts of p-aminophenylguanidine hydroiodide were weighed out, dissolved in the proper volumes of distilled water, and administered by subcutaneous injection. The changes in blood sugar were followed at definite intervals over a period of 24 hours, the Folin micro blood sugar method (15) being used.

In one series of experiments the hypoglycemic action of p-aminophenylguanidine hydroiodide was compared with that of neosynthalin (Lilly product). Some difficulty was experienced in getting the neosynthalin completely into solution in distilled water. The tablets were first thoroughly pulverized, then ground in a mortar with the desired volume of distilled water for about 30 minutes. The suspension was then filtered to remove the insoluble coating and the clear filtrate used in the experimentation.

In one set of experiments in order to estimate the toxicity of p-aminophenylguanidine hydroiodide at the dosages used, the amino acid nitrogen and urea nitrogen changes .in the blood, following injection, were observed, the methods of Folin (16) and Folin and Wu (17) being used for the determinations.

The results obtained in these preliminary experiments were as follows:

A. *Blood Sugar Changes.*—A decided drop in blood sugar was noted following each injection of *p*-aminophenylguanidine hydroiodide. The maximum hypoglycemia appeared to occur from 4 to 6 hours after the injection, and was not accompanied by convulsive symptoms in any case. Doses as low as 5 mg. per kilo produced a marked lowering of blood sugar. The data are recorded in Table I.

In comparison with neosynthalin *p*-aminophenylguanidine hydroiodide showed more consistent results, although a greater drop in blood sugar was observed with the former at 5 mg. per kilo injected at a concentration of 2.5 mg. per cc. It is to be

TABLE I.

Changes in Blood Sugar after Neosynthalin and p-Aminophenylguanidine Hydroiodide Injections.

Compound.	Dose.	Concentration of solution injected.	Blood sugar readings (mg. per 100 cc.).								
			Initial.	1½ hr.	2 hr.	3 hr.	4 hr.	6 hr.	8 hr.	10 hr.	24 hr.
	mg. per kilo	*mg. per cc.*									
Neosynthalin	5.0	2.5	110		92		69	89	94		105
"	5.0	2.5	100		97		94	83	95		100
$H_2NC_6H_4N_3CH_4 \cdot HI$	5.0	2.5	100		82		76	74	77		88
"	5.0	2.5	108		86		85	76	77		94
"	10.0	5.0	116		98		70	98	101		109
"	10.0	5.0	112		94		70	94	98		100
"	10.0	10.0	116	99		86		62		81	96

noted, however, that the neosynthalin results were in very poor agreement, and in one series produced only a slight drop in blood sugar. This result was in agreement with those reported by Bischoff (13). The comparative data for neosynthalin and *p*-aminophenylguanidine hydroiodide are shown graphically in Fig. 1, and the data for *p*-aminophenylguanidine hydroiodide at 10 mg. per kilo injected at a concentration of 5 and 10 mg. per cc., in Fig. 2.

B. *Apparent Toxicity.*—In each series the animals gave no visible symptoms of toxic effects, and no convulsions were produced in any case. Furthermore, all of the rabbits survived the experimentation and appear perfectly normal in health. These
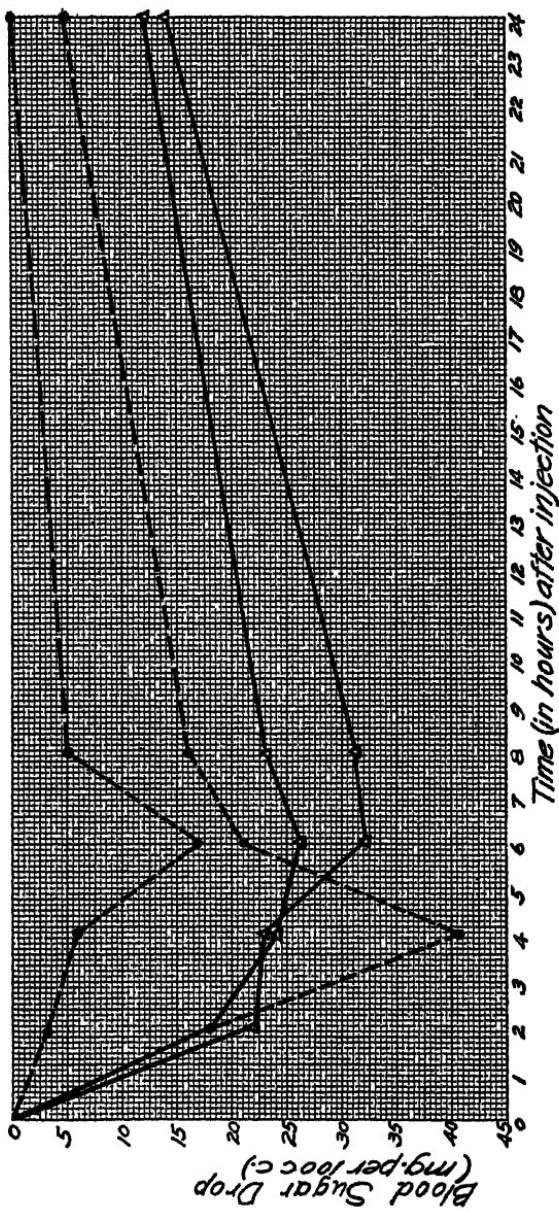
Fig. 1. Blood sugar curves following injection of neosynthalin ($\bullet$——$\bullet$) and $H_2NC_6H_4N_3CH_4 \cdot HI$ ($\triangle$——$\triangle$).
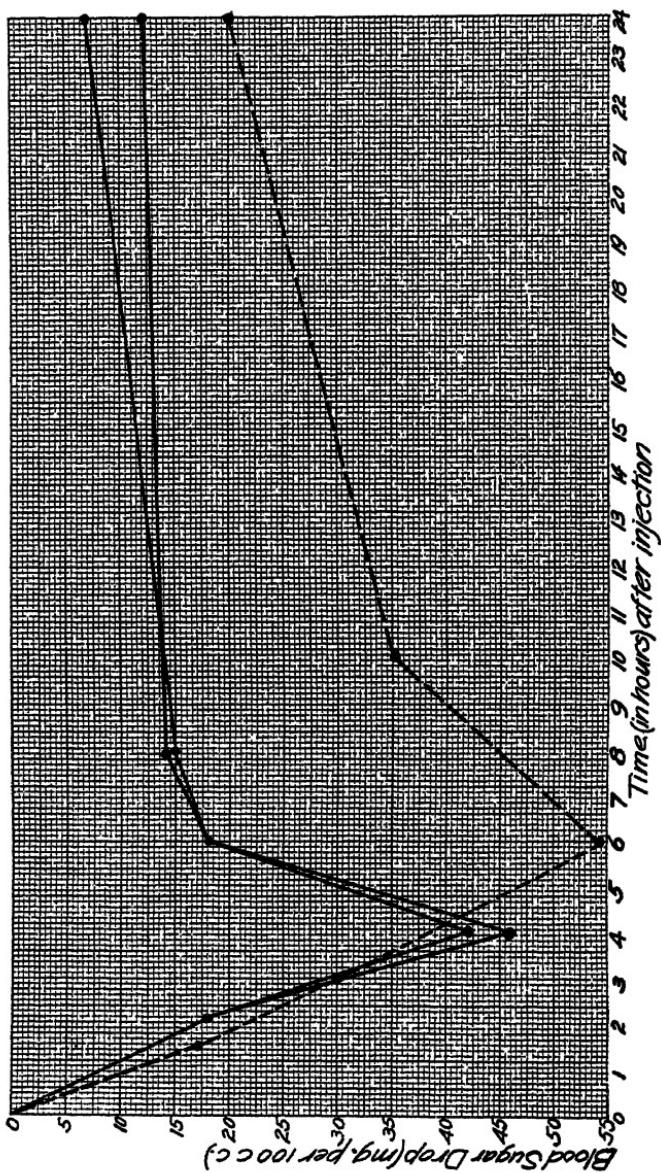
FIG. 2. Blood sugar curves for $H_2NC_6H_4N_3CH_4 \cdot HI$ at 10 mg. per kilo injected at a concentration of 5 mg. per cc. (●——●) and 10 mg. per cc. (●----●).

qualitative observations on the toxic effects of p-aminophenyl-guanidine hydroiodide in the doses used in these trials, appear to have been confirmed by the quantitative observations made upon the amino acid nitrogen and urea nitrogen of the blood in one set of experiments. Practically no change was noted in either amino acid nitrogen or urea nitrogen in this series.

It is fully realized that these results represent data from only three series of experiments performed upon normal rabbits. Therefore no speculation as to the action of p-aminophenylguani-dine hydroidide upon diabetic animals should be made. Furthermore, the possibility of affecting the thyroid metabolism by injected iodine has been considered, and it is planned to develop the preparation of both the sulfate and the chloride of p-aminophenylguanidine for use in future experimentation.

The author wishes to acknowledge with thanks the cooperation of Mr. T. B. Parks, who carried out the physiological part of this investigation. Thanks are due to Mr. B. Singerman for his assistance during the animal experimentation. The author is indebted to Eli Lilly and Company for the neosynthalin, and to the American Cyanamid Company for the thiourea used in this work.

SUMMARY.

1. The preparation of p-aminophenylguanidine hydroiodide has been described.

2. The solubility of p-aminophenylguanidine hydroiodide at 25° in several solvents has been determined.

3. p-Aminophenylguanidine hydroiodide has been shown to produce without noticeable toxic symptoms, a hypoglycemia when subcutaneously administered, without the appearance of hyperglycemia as is usually experienced with synthalin types.

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ARGININE METABOLISM.

I. THE RELATION OF THE ARGININE CONTENT OF THE DIET TO THE INCREMENTS IN TISSUE ARGININE DURING GROWTH.

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Of the twenty or more amino acids which occur in proteins only four have been definitely established as indispensable dietary components, namely, tryptophane, lysine, cystine, and histidine. Several others may be regarded at the present time as of uncertain significance inasmuch as the evidence is conflicting as to their nutritive importance. Of these, arginine is of particular interest. The wide distribution of this amino acid in nature suggests teleologically that it may be an essential component of the diet. Indeed, in certain lower animal forms it replaces creatine as a muscle component (*cf.* Ackermann, 1920, 1921, 1922). Furthermore, it is the only amino acid known to contain the guanidine group present in creatine and creatinine.

Attempts to associate arginine with the growth processes, however, have not yielded uniform results. The well known paper of Ackroyd and Hopkins (1916) appeared to indicate that histidine and arginine are interchangeable in metabolism. On the other hand, work in this laboratory (Rose and Cox, 1924, 1926) demonstrated clearly the indispensable nature of histidine, but showed that it cannot be replaced by arginine. More recently, Bunney and Rose (1928) have presented additional data regarding the relation of arginine to growth. After the precipitation of arginine

as the flavianate from a concentrated solution of hydrolyzed casein, the resulting mixture of amino acids supports excellent growth. When the amino acids compose 12 per cent of the ration, the animals grow at practically normal speed. When the nitrogenous portion of the diet is decreased to 9 per cent, the increase in body weight occurs at about half the normal rate. That the less rapid growth under the latter circumstance is not to be ascribed to a deficiency of arginine is shown by the fact that the addition to the diets of the amino acid in question entirely fails to accelerate growth.

In experiments of this sort, involving the removal of an amino acid from a hydrolyzed protein, the possibility exists that the traces which fail to be precipitated, together with the quantities which are introduced into the diet unavoidably in the vitamin supplement, may be sufficient to meet the growth requirements of the animals. With these facts in mind we have attacked the problem by an entirely different method, the results of which are outlined below.

<div align="center">EXPERIMENTAL.</div>

The procedure involves a comparison of the arginine intake of growing rats on an arginine-low diet and the increments in tissue arginine, in order to determine whether the latter may be accounted for by the amounts of the amino acid in the basal ration and vitamin supplement. For this purpose, hydrolyzed casein was rendered as nearly devoid of arginine as possible, and was incorporated in a diet which was administered *ad libitum*. At the beginning of the experiments, litter mates of the animals employed in the growth studies were killed and subjected *in toto* to analysis for arginine. The other members of each litter were killed and analyzed after they had received the experimental diet for a period of 64 days. In the meantime they had gained 73 to 113 gm. each.

Obviously, the first prerequisite for such an investigation is a satisfactory method for the estimation of arginine in the food and tissues. The method of Bonot and Cahn (1927) was found to be best adapted to these purposes.[1] The principle of this method is

[1] Since this investigation was completed the recent paper of Hunter and Dauphinee (1929-30) has appeared in which is described the determination of arginine by the successive use of arginase and urease.

the transformation of arginine into ornithine and urea by the use of arginase, and the gravimetric estimation of the urea as the xanthydrol derivative. The large molecular weight of the resulting compound, composed of two molecules of xanthydrol and one of urea, renders the reaction a suitable one for the estimation of small amounts of arginine. A number of preliminary experiments were conducted in order to establish the best conditions for the application of the procedure to mixed tissues. Inasmuch as the validity of our conclusions depends upon the reliability of the method of analysis, the modified technique as finally adopted is described in detail below.

Estimation of Arginine.—Each animal was weighed, chloroformed, and the contents removed from its alimentary tract. The weight of the total tissues minus the alimentary residues was then noted, and the whole was passed through a meat chopper at least six times. Between each grinding the material was thoroughly mixed. In this fashion no difficulty was encountered in securing a sufficiently uniform product despite the presence of skin and skeletal structures. 20 gm. were weighed as rapidly as possible into a 500 cc. round bottom flask, and treated with 200 cc. of 30 per cent hydrochloric acid. The flask was fitted to a condenser with a ground glass connection, and the contents were boiled for 24 hours. The solution was then transferred quantitatively to a 50 cc. Claisen flask, and concentrated under reduced pressure on a steam bath until a syrup remained. To aid in the removal of the hydrochloric acid, the evaporation was repeated twice after dissolving the residue each time in 200 cc. of distilled water. The material was dissolved in 100 cc. of water and transferred quantitatively to a 300 cc. Erlenmeyer flask. 2 gm. of acid-washed norit were added, and the mixture was heated for 2 to 3 hours on a steam bath. The whole was then transferred to a fluted filter paper, and the residue was washed repeatedly with hot water. The filtrate and washings were placed in a 250 cc. volumetric flask, diluted to the mark with distilled water, and preserved with toluene.

Two 100 cc. portions of the above solution were pipetted into 300 cc. Erlenmeyer flasks, and evaporated on a hot plate to a volume of about 10 cc. The samples were transferred to 125 cc. centrifuge tubes and diluted to 20 cc. 2 drops of phenolphthalein were added to the contents of each tube. To one, 10 per cent

sodium hydroxide was added until a pink color persisted after mixing. The precipitate which separated at this point was centrifugated, and the tube was placed in a comparator block for the adjustment of the pH. This was accomplished by the addition of sodium hydroxide until the color exactly matched that of the second sample used as a blank and placed in series with a tube containing 25 cc. of a standard sodium hydroxide-glycocoll buffer of pH 9.7 to which 2 drops of phenolphthalein had been added. 3 cc. of arginase solution (described below) were added to the unknown, and the resulting mixture was placed in an incubator at 40° for 48 hours. At the expiration of this time, the solution was treated with 30 cc. of glacial acetic acid. After standing for 30 to 60 minutes, the tube and contents were centrifugated, and the clear supernatant fluid was decanted into another 125 cc. centrifuge tube. The precipitate was washed by the addition of 40 cc. of glacial acetic acid, thoroughly mixed, and centrifugated. The supernatant solution was added to the first. The urea was then precipitated by the addition of 5 cc. of a 10 per cent solution of xanthydrol in methyl alcohol, the whole being allowed to stand for 10 to 15 hours.[2] The precipitate of dixanthydrylurea was centrifugated, and the supernatant liquid was poured through a weighed fritted glass bottom crucible. The precipitate was washed by centrifugation with two 10 cc. portions of methyl alcohol saturated with dixanthydrylurea, and transferred quantitatively to the crucible. The latter was then dried at 105° for 2 hours, cooled in a desiccator, and weighed. The weight of the dixanthydrylurea multiplied by 0.414 gives the amount of arginine in the 100 cc. aliquot.

The *arginase* solution was prepared according to the method of Hunter and Dauphinee (1924–25). For this purpose, the liver was removed from a fasting dog, and was washed practically free of blood by perfusion with 0.9 per cent sodium chloride solution. 300 gm. of the moist tissue were passed through a meat chopper,

[2] In several tissue analyses blanks were run without the arginase digestion in order to provide a correction factor for the preformed urea of the tissues. In none of these tests, however, were precipitates secured after the xanthydrol treatment. Evidently the trace of urea present in the tissues is largely or completely decomposed by the prolonged hydrolysis and repeated evaporations with hydrochloric acid.

and ground in a mortar with 300 gm. of sand and 300 cc. of glycerol. A little toluene was added, and the whole was allowed to stand overnight. It was then shaken for 5 hours in a shaking machine. The bulk of the sand was removed by filtration through several layers of cheese-cloth. $\frac{1}{4}$ volume of toluene was added to the

TABLE I.

Arginine Estimation in Solutions of Pure Amino Acid and in Hydrolyzed Proteins.

Material.	Weight of sample.	Arginine added.	Dixant-hydryl-urea.	Arginine.		Recovery.	Arginine content.
				Theory.	Found.		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
Arginine	35		68.0	28.9	28.2	97.6	
monohydro-	35		70.0	28.9	29.0	100.3	
chloride.	35		71.8	28.9	29.7	102.8	
	35		69.2	28.9	28.6	99.0	
	42		82.0	34.7	33.9	97.7	
	42		85.0	34.7	35.2	101.4	
	120		238.0	99.2	98.5	99.3	
	120		242.0	99.2	100.2	101.0	
Gelatin	3000	0	205*		212		7.07
(Difco).	5000	0	335*		347		6.94
	8000	0	272†		563		7.04
	3000	82.7	292*	293	302	103.1	
	4000	82.7	358*	364	371	101.9	
	2000	124.0	252*	264	261	98.9	
Casein (Har-	5000	0	157*		162		3.24
ris).	5000	82.7	235*	245	243	99.2	
	5000	82.7	238*	245	246	100.4	
	4000	82.7	202*	212	209	98.6	
	3000	124.0	215*	221	223	100.9	

* From an aliquot representing two-fifths of the sample.
† From an aliquot representing one-fifth of the sample.

filtrate, and mixed by shaking. On centrifugation, a semisolid mass containing fat accumulated at the surface, and the residue of sand settled to the bottom. The fluid between these two layers was employed as the enzyme solution. Yield, about 300 cc.

The *xanthydrol* was made by reducing xanthone with sodium amalgam in ethyl alcohol (Holleman, 1927). A freshly prepared

10 per cent solution of xanthydrol in methyl alcohol served as the reagent for urea precipitation. Inasmuch as both the solid and solution appear to deteriorate on standing, new preparations were always employed.

The reliability of the above method for the estimation of arginine was tested by applying it to solutions of arginine monohydrochloride, and to casein and gelatin hydrolysates with and without added arginine. With the arginine monohydrochloride solutions it was necessary to add a buffer before introducing the enzyme. 5 cc. of a sodium hydroxide-glycocoll mixture (pH 9.7)

TABLE II.

Arginine Content of the Amino Acid Mixture and Yeast Extract.

Material.	Weight of sample.	Arginine added.	Dixant-hydryl-urea.	Arginine.		Recovery.	Arginine content.
				Theory.	Found.		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
Arginine-low	3000	0	28.0		11.6		0.39
amino acid	4000	0	37.5		15.5		0.39
mixture.	4000	30	113.0	45.5	46.8	102.9	(0.42)*
	3000	30	103.0	41.6	42.6	102.4	(0.42)*
Yeast extract	5000	0	127.0†		131.4		2.63
(Harris).	5000	0	125.0†		129.4		2.59
	5000	50	178.0†	180.4	184.2	102.1	(2.68)*
	3000	82.7	153.0†	160.9	158.4	98.4	(2.52)*
	4000	82.7	180.0†	187.0	186.3	99.6	(2.59)*

* The figures in parentheses represent the percentage of arginine in the samples after deducting the added arginine from the arginine found.

† From an aliquot representing two-fifths of the sample.

were employed for this purpose. With the protein and tissue hydrolysates, the buffering effect of the concentrated mixture of amino acids is adequate. In Table I are summarized the results of these preliminary tests. The data show that the method yields recoveries of arginine in pure solutions within 3 per cent of the theory. The values for the arginine content of gelatin and casein, namely, 7.02 and 3.24 per cent, are lower than the generally accepted values of 8.2 and 3.81 per cent. These differences doubtless are to be accounted for by the fact that the proteins employed by us were air-dried commercial products which had not been sub-

jected to special purification or drying procedures preliminary to analysis. Arginine added to these hydrolyzed proteins was recovered as accurately as in pure solutions of the amino acid. In view of the complicated nature of the procedure, the errors are not greater than might be expected. Certainly, the method would appear to be satisfactory for the purposes in question, inasmuch as only large differences between the arginine intake and content of tissues could be regarded as significant.

The arginine-low amino acid mixture, the preparation of which is described below, and yeast extract (Harris) employed in the diet were also analyzed. The recovery of arginine added to these materials was likewise determined. The data are presented in Table II. The surprizingly high percentage of arginine in the yeast extract is worthy of note. The arginine-low amino acid

TABLE III.

Comparison of the Flavianic Acid and Arginase Methods for the Estimation of Arginine in Mixed Rat Tissues.

Sample.	Arginine content.	
	Flavianic acid method.	Arginase method.
	per cent	*per cent*
A	1.31	1.43
B	1.35	1.47
C	1.27	1.26

mixture showed the presence of approximately 0.4 per cent of the amino acid.

As a final test of the method, three samples of mixed rat tissues were subjected to analysis for arginine by the flavianic acid procedure of Kossel and Staudt (1926) and the modified technique of Bonot and Cahn. The comparative results are summarized in Table III. Of the three samples, one (Sample C) showed a close agreement by the two methods. With the other two samples, the flavianic acid method yielded values 0.12 per cent lower than those secured by the use of arginase. Our experience with the flavianic acid method has led us to believe that it is less reliable than the arginase procedure. But even if one assumes that the lower values for arginine in Table III are the more accurate, this assumption would not alter the interpretation of the data to be

described below. The differences between the dietary arginine and arginine increments in the tissues of rats, as will be shown later, are much too large to be explained by variations such as are shown in Table III. We feel, therefore, that the arginase method certainly is not subject to gross errors which would invalidate our conclusions.

Preparation of Arginine-Low Amino Acid Mixture.—In a number of preliminary experiments it appeared that the flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) method of Kossel and Gross (1924) accomplishes a more complete removal of arginine

TABLE IV.
*Composition of Diet.**

	gm.
Arginine-low amino acid mixture.....................	11.1
Cystine...	0.3
Tryptophane..	0.2
Histidine...	0.4
Dextrin..	43.0
Sucrose..	15.0
Lard...	19.0
Cod liver oil..	5.0
Salt mixture.†......................................	4.0
Agar...	2.0
	100.0

* Vitamin B factors were supplied in the form of two pills daily each containing 75 mg. of Harris yeast extract, and administered at approximately 12 hour intervals.
† Osborne and Mendel (1919).

from a hydrolyzed protein than does the older silver procedure. Therefore, the former was employed in this investigation. For this purpose, 3 kilos of casein were refluxed on a sand bath for 16 hours with a mixture of 3750 cc. of concentrated sulfuric acid and 11,250 cc. of distilled water. After dilution, the mixture was treated with finely powdered barium hydroxide until only a trace of sulfuric acid remained. The precipitate was filtered off and washed twice with hot water. The combined filtrate and washings were then evaporated *in vacuo* to a volume of 8 liters, and treated with 600 gm. of flavianic acid dissolved in 300 cc. of hot water.

The mixture was stirred vigorously until the arginine salt began to crystallize out, and was allowed to remain in an ice box for 4 days. The arginine flavianate was then filtered off, and the filtrate was treated with barium hydroxide for the removal of the excess flavianic acid. The resulting filtrate was cautiously treated with dilute sulfuric acid until filtered samples showed the complete absence of both the barium and sulfate ions. The final solution was evaporated *in vacuo* to a thick paste, and dried at 80° in a

TABLE V.

Body Weight Increase and Arginine Intake.

Rat No. and sex.	Total increase in weight.	Total food consumption.	Arginine intake.*		
			In basal diet.	In vitamin pills.	Total.
	gm.	*gm.*	*mg.*	*mg.*	*mg.*
1198♂	106	440	205	257	462
1199♂	93	399	186	257	443
1200♀	113	431	201	257	458
1201♀	110	449	209	257	466
1202♀	96	417	194	257	451
1203♀	110	463	216	257	473
1220♂	101	400	186	257	443
1221♂	105	412	192	257	449
1222♂	102	465	217	257	474
1223♀	95	450	210	257	467
1224♀	82	499	233	257	490
1225♀	81	480	224	257	481
1226♀	73	449	209	257	466
1227♀	99	534	249	257	506
1228♀	91	448	209	257	466

* See Table II for results of analyses of amino acid mixture and yeast extract.

vacuum oven. The resulting material was ground, and passed through a 40 mesh sieve. The yield amounted to 1325 gm. The final product contained a trace of flavianic acid. Inasmuch as the latter is a non-toxic dye used as a food color the presence of a trace in the amino acid mixture is of no consequence.

The composition of the diet is shown in Table IV. The results of the experiments are summarized in Tables V to VII inclusive. The growth of the rats is demonstrated in Charts I and II. *In*

TABLE VI.

Arginine Content of Tissues.

Litter	Rat No. and sex*	Initial body weight.		Final body weight.		Total arginine content.				Arginine increment.
		Live.	Minus alimentary contents.	Live.	Minus alimentary contents.	Initial.	Final.	Initial.	Final.	
		gm.	gm.	gm.	gm.	per cent	per cent	mg.	mg.	mg.
A	1♂	64	60			1.22				
	2♀	66	62			1.28				
	3♂	64	59			1.28				
	1198♂	61	57	167	161	1.26	1.40	718	2254	1536
	1199♂	69	65	162	158	1.26	1.39	819	2196	1377
	1200♀	61	57	174	163	1.26	1.41	718	2298	1580
B	4♀	63	61			1.23				
	5♀	53	51			1.24				
	1201♀	57	55	167	161	1.24	1.44	682	2318	1636
	1202♀	65	63	161	155	1.24	1.39	781	2155	1374
	1203♀	61	59	171	165	1.24	1.42	732	2343	1611
C	6♂	57	52			1.23				
	7♂	71	65			1.31				
	1220♂	57	52	158	149	1.27	1.39	660	2071	1411
	1221♂	61	56	166	159	1.27	1.46	711	2321	1610
D	8♂	80	74			1.25				
	9♀	72	68			1.28				
	1222♂	72	66	174	163	1.27	1.41	838	2298	1460
	1223♀	66	62	161	155	1.27	1.42	787	2201	1414
E	10♀	85	78			1.26				
	11♀	92	84			1.27				
	1224♀	91	83	173	165	1.27	1.46	1054	2409	1355
	1225♀	91	83	172	165	1.27	1.49	1054	2459	1405
F	12♀	110	98			1.28				
	13♀	102	90			1.31				
	1226♀	102	90	175	164	1.30	1.42	1170	2329	1159
	1227♀	103	91	202	194	1.30	1.67	1183	3240	2057
	1228♀	96	84	187	178	1.30	1.50	1092	2670	1578

* Rats 1 to 13 inclusive are the controls which were killed and analyzed at the beginning of the experiments.

calculating the arginine intakes of our animals we employed the highest figures secured in the analyses of the amino acid mixture and yeast extract (Table II), namely, 0.42 and 2.68 per cent respectively. Thus *the figures in Table V represent maximum values.* The data make evident the fact that more than half of the arginine ingested originated in the yeast extract. The total intakes of the amino acid during the 64 day period ranged from 443 to 506 mg., the latter being the intake of Rat 1227 which consumed an exceptionally large amount of the basal diet.

TABLE VII.

Apparent Arginine Synthesis.

Rat No. and sex.	Arginine increment in tissues.	Total arginine intake.	"Arginine synthesis."
	mg.	*mg.*	*mg.*
1198♂	1536	462	1074
1199♂	1377	443	934
1200♀	1580	458	1122
1201♀	1636	466	1170
1202♀	1374	451	923
1203♀	1611	473	1138
1220♂	1411	443	968
1221♂	1610	449	1161
1222♂	1460	474	986
1223♀	1414	467	947
1224♀	1355	490	865
1225♀	1405	481	924
1226♀	1159	466	693
1227♀	2057	506	1551
1228♀	1578	466	1112

In Table VI are shown the increments in tissue arginine which occurred incidental to growth. Rats 1 to 13 inclusive are the controls which were analyzed at the beginning of the experiments. The animals which were permitted to grow are numbered Rats 1198 to 1203 and Rats 1220 to 1228 inclusive. The average weight of the alimentary contents in the controls of each litter was assumed to represent the initial weight of the alimentary residues in the growing members of that litter. In a similar fashion, the average percentage of arginine in the controls was used in calculating the initial arginine content of the litter mates which received the diet.

CHART I.
Growth on Arginine-low Diet.

Rats: 1198-1200, Litter A
      1201-1203, Litter B
      1220-1221, Litter C

(158)
(171)
(174)
(167)
(166)
(167)
(161)
(162)

1220 ♂ (57)

1203 ♀ (61)

1200 ♀ (61)

1201 ♀ (57)

1221 ♂ (61)

1198 ♂ (61)

1202 ♀ (65)

1199 ♂ (69)

↑ 30 gm. ↓

← 20 days →
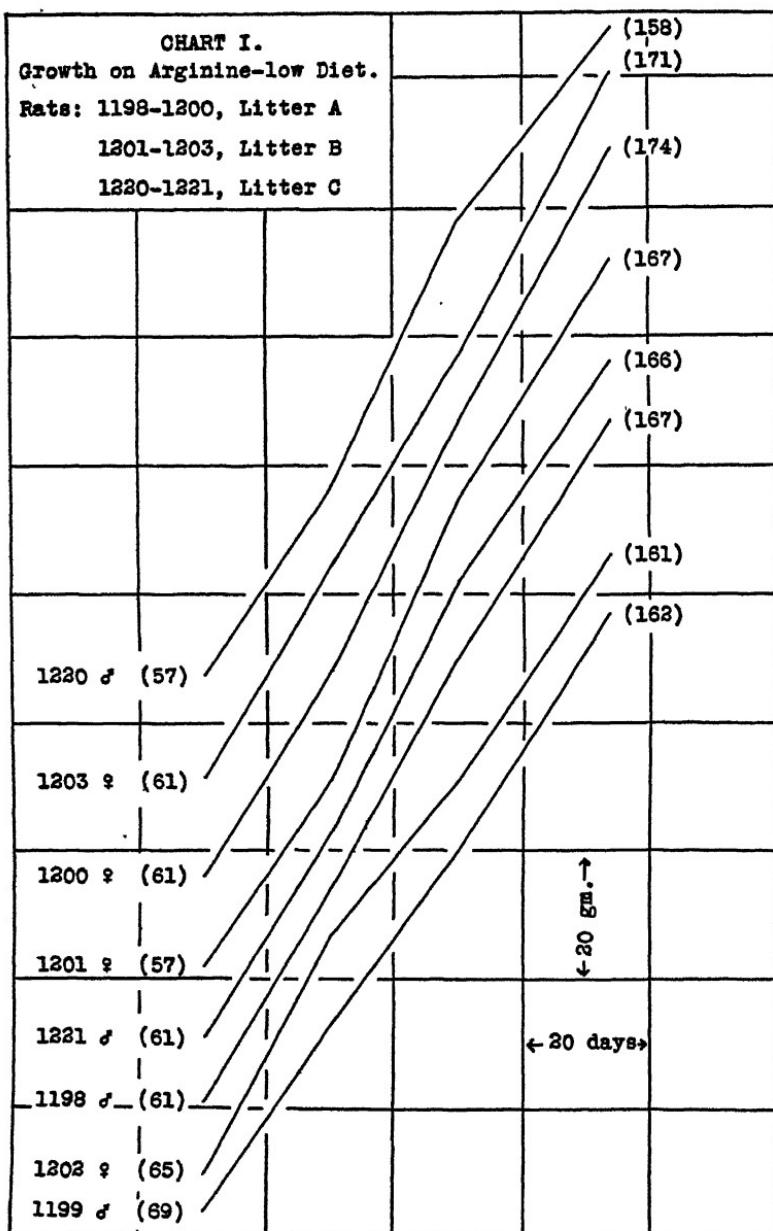
CHART I. The numbers in parentheses denote the initial and final weights of the rats.

CHART II.
Growth on Arginine-low Diet.
Rats: 1222-1223, Litter D
        1224-1225, Litter E
        1226-1228, Litter F

(174)
(202)
(161)
(173)
(172)
(187)
(175)

1222 ♂ (72)
1227 ♀ (103)
1223 ♀ (66)
1224 ♀ (91)
1225 ♀ (91)
1228 ♀ (96)
1226 ♀ (102)

←20 gm.→

←20 days→
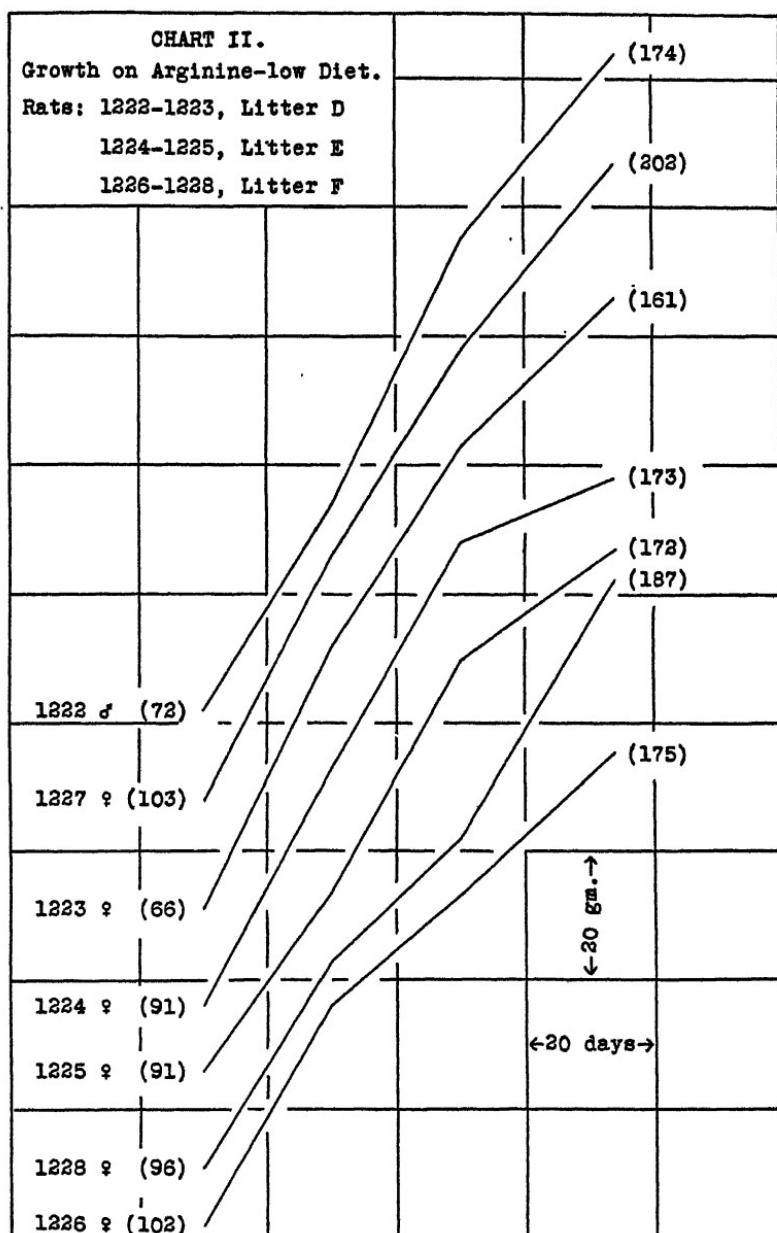
CHART II. The numbers in parentheses denote the initial and final weights of the rats.

It is to be noted that the *percentage* of arginine in the animals at the expiration of the growth period was invariably larger than in the controls at the beginning of the experiments.   Probably this is to be accounted for by the fact that water and the skeletal structures constitute a smaller proportion of the total body weight of older (larger) rats than of younger ones.

The figures in the last column of Table VI, representing the increments in arginine, demonstrate that each of the fifteen animals increased its content of the amino acid by a value ranging from 1159 to 2057 mg.   The three members of Litter F show the greatest variation of the series.   The other twelve animals yielded arginine increments of 1355 to 1636 mg., and the latter values are in most cases rather closely proportional to the total gains in body weight.

In Table VII are compared the arginine intake and the increase in tissue arginine.   The data show that *without exception the increase in tissue arginine is 2 to 3 times as large as can be accounted for by the total arginine consumed in the diet.*   While one would not be justified, perhaps, in view of the complicated nature of the analytic procedure, in regarding the figures as representing *absolute amounts*, nevertheless the occurrence of errors sufficiently large to invalidate our results appears very improbable.   Therefore, even a conservative interpretation of our findings would seem to warrant the conclusion that arginine may be synthesized by the organism of the rat, and in this species at least is not an indispensable dietary component.

SUMMARY.

Comparative studies have been made of the increase in tissue arginine incidental to growth, and the arginine intake of rats upon an arginine-low diet.   The results show that under the conditions employed, the increments in tissue arginine are 2 to 3 times as large as may be accounted for by the arginine ingestion.   Evidently arginine may be synthesized by the organism of the rat, and in this species is not an essential component of the diet.

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THE GASOMETRIC DETERMINATION OF CYSTEINE AND CYSTINE.

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In this paper is described an adaptation of the gasometric technique of Van Slyke to the determination of cystine, which it is believed presents some advantages over methods previously employed in the quantitative estimation of this amino acid. Because of the importance of cystine as an essential constituent of food and protoplasm, its probable rôle in biological oxidation, and its relation to glutathione and perhaps insulin, it is believed that any improvement in the method of cystine assay is of real value. The method to be described avoids the danger of destruction incident to alkaline hydrolysis, the errors due to solubility in isolating and weighing the amino acid itself, and is applicable to the determination of cystine in pure proteins and to its liberation during protein hydrolysis.

The method depends upon the fact that cystine may be reduced quantitatively to cysteine by nascent hydrogen. The cysteine is then oxidized back to cystine by a known amount of an appropriate oxidizing agent, and the excess of the latter determined gasometrically in the Van Slyke apparatus by its reaction with hydrazine, much as in the Van Slyke gasometric sugar determination (1).

The two points of difficulty lie in finding a reducing agent which will reduce the cystine rapidly and quantitatively and which may then be readily removed. The second difficulty is in developing an oxidizing agent which is so specific for cysteine that other reducing agents like tyrosine and tryptophane, uric acid, and creatinine, will not interfere.

After trial of a number of reducing agents, nascent hydrogen evolved from Devarda's alloy in acid solution appears to be ideal.

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Pure cystine is reduced quantitatively to cysteine under the conditions described below in 10 minutes, and there is no problem of removing an excess of the reducing agent, beyond filtering off the the excess metal.

The question of finding an oxidizing agent so specific as to oxidize cysteine back to cystine without at the same time oxidizing other components likely to be present in biological material, seems to be satisfactorily answered by the use of iodine in strongly acid solution, as was used in the titration method of Teruuchi and Okabe (2).

A series of preliminary experiments established the fact that an exact relationship exists between iodine taken and nitrogen liberated from hydrazine in the gasometric apparatus. This is shown in the following figures.

Iodine. cc.	Δp mm.
1.0	16.2
2.0	32.7
4.0	66.6
6.0	101.5
8.0	131.5
10.0	163.8

A second series of experiments made with cysteine established the fact that cysteine is oxidized by a known amount of iodine in acid solution, and the excess iodine can then be determined with precision. It was also established that cystine was reduced to cysteine by nascent hydrogen and that the cysteine so produced and determined gave a precise estimate of the cystine taken. This is shown in the following figures.

Standard solution. cc.	Cysteine. Δp mm.	Cystine. Δp mm.
0.2	13.4	12.0
0.4	21.6	20.0
0.6	31.8	33.5
0.8	43.6	43.3
1.0	54.4	54.0

A study of the time required for the oxidation of cysteine by iodine established the fact that this reaction was complete in something less than 5 minutes, and that there was no further loss of

iodine in prolonged contact, which confirms our qualitative experiments to show that the oxidation goes no further than the reaction SH→SS. There is, however, a slow increase in the amount of iodine present after an hour. This is due to the oxidation of hydriodic acid by the oxygen of the air, and therefore only fresh mixtures should be used.

The time required for the reduction of cystine to cysteine by nascent hydrogen was found to be less than 10 minutes under the conditions of the method and the amounts of cystine taken.

Finally, the specificity of iodine in acid solution as oxidant for cysteine was established by using various amino acids and other substances, such as urea, uric acid, creatinine, and creatine in

TABLE I.

Reducing Power of Several Amino Acids.

	Δp at 25°.		Δp at 25°.
	mm.		*mm.*
Cysteine-HCl............	55.6	Alanine...............	+0.6
Cystine.................	−0.4	Leucine...............	+0.8
Tyrosine...............	−0.9	Glutamic acid..........	−1.2
Tryptophane...........	+0.8	Aspartic "	−0.2
Histidine...............	−0.1	Phenylalanine..........	+0.6
Glycine.................	−0.1	Valine................	+0.8

amounts approximately equivalent to the amount of cysteine in the standard solution. None of the substances tried was found to reduce the iodine solution under the conditions used, and therefore we may conclude that the reagent is highly specific for the sulfhydryl group of cysteine. The figures are shown in Table I.

Method.

Reagents.

Iodine Solution.—30 gm. of potassium iodide are dissolved in about 100 cc. of water and 10 gm. of iodine crystals are added. After the iodine has dissolved, dilute to 4 liters.

Hydrazine Solution.—Saturate distilled water with hydrazine sulfate and mix with an equal volume of sodium hydroxide solution (200 gm. per 500 cc.).

Hydrochloric Acid.—Concentrated; specific gravity 1.18.

Procedure for Preformed Cysteine.

2 cc. of a solution containing 1 to 4 mg. of cysteine in HCl (1 to 8 N) are measured into a 15 cc. centrifuge tube. 1 cc. of concentrated HCl and 2 cc. of iodine solution are added and the tube inverted several times to mix. After the mixture has come to room temperature the tubes are vigorously shaken to saturate them with air. 1 cc. of this mixture is then drawn into a glass stop-cock pipette[1] recommended by Van Slyke and Neill, and introduced under mercury into the chamber of the manometric apparatus which has previously been charged with 2 cc. of hydrazine solution. The leveling bulb is lowered and while the mixture is descending in the chamber, the upper cock is sealed with mercury. The chamber is rinsed by raising and lowering the bulb several times and then evacuated to the 50 cc. mark and shaken for 2 minutes.[2] The pressure of the gas (p_1) is then read at a volume of 0.5 cc. This gas consists of air from the reagents to the extent of about 100 mm. of pressure; the rest is nitrogen from the hydrazine reaction. A blank containing 2 cc. of HCl instead of cysteine solution is analyzed at the same temperature giving p_0, which includes the air in reagents and nitrogen liberated by all the iodine employed. Subtracting p_1 from p_0, one gets the value Δp which is strictly proportional to the amount of cysteine in the sample.

Procedure for Cystine.

About 5 cc. of a solution containing less than 10 mg. of cystine in HCl (1 to 8 N) are placed in a 50 cc. flask and a few gm. of Devarda's alloy added. The flask is closed with a stopper carrying a piece of capillary tubing about 2 inches long. Let stand for

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[1] In filling the special pipette, it has been found convenient to use a mouthpiece made of a small 1 inch funnel with a short stem, which is covered with a piece of rubber tubing. The delivery end of the pipette is pressed against the funnel which is held in the mouth and the solution drawn up. This prevents the entrance of the acid solution into the mouth.

[2] A simple mercury time switch built on the principle of the old fashioned sand hour-glass was designed to obviate the use of a clock for timing the shaking interval. Mercury is allowed to flow from one bulb to another through a capillary tube carrying two electrodes. As long as the mercury is flowing electrical connection is made and the chamber is shaken. The device is mounted to facilitate inversion.

10 to 15 minutes with occasional shaking, then filter off the excess alloy. 2 cc. of this solution are treated exactly as the cysteine solution of the previous section. The blank in this case should be treated with alloy just as the cystine solution was treated.

Calculations.—Since the difference in pressure, Δp, observed between the blank and the sample is proportional to the quantity of cysteine present, one can calculate factors for converting mm. of pressure into mg. of cysteine or cystine. From a large number of determinations we have found Δp equals 48.1 ± 0.7 mm. at $25°$,

TABLE II.

Factors for Converting Mm. of Δp into Mg. of Cysteine and Cystine.

$$F = \frac{1}{P} \times \frac{273 + 25}{273 + t}.$$

$P = \Delta p$ per mg. of cysteine or cystine $= 12.62$ mm. and 12.84 mm. respectively.

t	Cysteine.	Cystine.
°C.		
20	0.0813	0.0799
21	0.0811	0.0797
22	0.0808	0.0794
23	0.0805	0.0792
24	0.0803	0.0789
25	0.0801	0.0787
26	0.0797	0.0786
27	0.0794	0.0779
28	0.0792	0.0778
29	0.0787	0.0776
30	0.0783	0.0773

which is equivalent to 3.85 mg. of cysteine or 3.79 mg. of cystine. The factors for various temperatures are given in Table II.

Mixtures of cysteine and cystine have been analyzed with satisfactory precision; for example, one containing 2.31 mg. of cysteine and 1.51 mg. of cystine gave 2.32 and 1.52 mg. respectively. When cystine was added to protein digests, the recovery was 96 to 100 per cent.

Cystine of Proteins.

1 gm. of protein was heated on a reflux apparatus for 15 hours with 20 cc. of 20 per cent HCl. The hydrolyzed protein was washed

into a 25 cc. volumetric flask with 5 cc. of HCl.   10 cc. were then
treated with Devarda's alloy as described above and 2 cc. used for
analysis.   Table III shows how the results compare with the
figures given by Teruuchi and Okabe and by Folin on similar
material.

DISCUSSION.

The present method has some advantages over those now in
use.   Colored solutions or turbidities do not influence the results
at all.   One can use partially hydrolyzed protein with equal suc-

TABLE III.

Cystine Content of Proteins.

Material.	Okabe method.	Folin method.	Gasometric method.
	per cent	*per cent*	*per cent*
Serum albumin....................	1.58	6.04	3.94*
Egg white........................	2.01		2.07†
" albumin....................		1.22	1.58*
Fibrin..........................	1.48	3.50	1.38
Gelatin...........................	0.04	0.20	0.00
Wool............................	9.12	7.80	10.28
Edestin..........................	1.13	1.35	1.75
Casein...........................	0.33	0.30	0.94

    * Samples were prepared by Dr. F. Holford of the bacteriology depart-
ment of the University of Wisconsin and kindly donated.
    † Merck's egg albumin.

cess and therefore the method may be used in enzyme studies.
It is simpler and more rapid than others and the accuracy is as
good as with any method in use at present.

SUMMARY.

A method for the determination of cysteine and cystine is
described which depends upon the reducing properties of the sulf-
hydryl group when oxidized in acid (2 N) by a solution of iodine
in potassium iodide.   A known excess of iodine is allowed to react
with cysteine and the excess is determined gasometrically by a
hydrazine titration in the apparatus of Van Slyke and Neill.   The
method is simple, rapid, and accurate to about 4 per cent.

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GASOMETRIC DETERMINATION OF OXYGEN AND CARBON MONOXIDE IN BLOOD.

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Van Slyke and Neill (1), in their original description of the manometric blood gas apparatus, described a method for the determination of O_2 and CO which was sufficiently exact for most purposes. However, estimation of the CO was less exact than that of O_2. While O_2 was determined by specific absorption with $Na_2S_2O_4$ solution, the CO had to be estimated by subtracting from the residual CO + N_2 a value of 1.2 or 1.4 volume per cent corresponding to the mean N_2 content of blood. CO could not be determined by absorption, because no suitable absorbing solution was available which did not form an unmanageable clot when mixed with the blood in the extraction chamber. The procedure by which CO is estimated by subtraction of the mean N_2 content of blood from the CO + N_2, is definitely less exact than one in which CO could be determined by direct absorption. Furthermore, the estimation by subtraction of the mean N_2 is not valid for blood saturated under experimental conditions with inert gases other than air at atmospheric pressure.

A technique which made possible the precise determination of CO by direct absorption was later devised by Van Slyke and Robscheit-Robbins (2) who used the Harington-Van Slyke (3) modification of the extraction chamber of the manometric apparatus. At the bottom of this extraction chamber there is an added cock, by means of which the chamber and the gases in it can be washed with successive portions of cleaning and absorbing solutions. The blood could be washed out and the CO measured by absorp-

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tion with cuprous chloride solution. The results were highly exact, but the numerous washings made the procedure rather long, 40 to 50 minutes being required for an analysis. Also, an inconvenience was introduced in this analysis, with the necessity for using the special Harington-Van Slyke extraction chamber, while all other analyses described for the manometric apparatus can be carried out with the simpler Van Slyke-Neill chamber.

In the present paper a procedure is described in which determination of CO by absorption is accomplished in analyses made with the Van Slyke-Neill chamber. The mixture of O_2 + CO + N_2 extracted from blood is removed to a micro-Hempel pipette, where the O_2 is absorbed, by a technique similar to that employed previously for manometric determination of amino nitrogen (4). The extraction chamber is then washed free of blood, and the gases are returned for completion of the analysis. The procedure equals in accuracy the Van Slyke-Robscheit-Robbins method, is less laborious, and can be carried through in 25 to 30 minutes.

Description of Method.

Reagents.

Acid Ferricyanide Reagent.—This is prepared for use each day by mixing equal parts of the following two solutions; (a) 32 gm. of potassium ferricyanide and 8 gm. of saponin dissolved in water to make 1 liter of solution, (b) 8 cc. of concentrated lactic acid (sp. gr. 1.20), diluted to 1 liter.

Alkaline Pyrogallate.—15 gm. of pyrogallic acid in 100 cc. of a saturated solution of KOH (sp. gr. about 1.55). This absorbent is kept under paraffin oil in a stoppered bottle and is not used until 3 weeks after preparation. If kept confined under oil in the modified Hempel pipette used for this work, one portion of pyrogallol solution may be used for 30 to 40 analyses.

Air-Free N Sodium Hydroxide.—Approximately 40 gm. of NaOH per liter solution. This is extracted air-free for use daily, and kept under oil in a calcium chloride tube ((1) p. 534).

Glycerol-Salt Solution.—One volume of glycerol is mixed with 3 volumes of saturated NaCl solution.

Winkler's Cuprous Chloride Solution.—200 gm. of CuCl, 250 gm. of NH₄Cl, and 750 cc. of water. The addition of a few gm. of

pure metallic copper serves to keep the CuCl in reduced state. This solution is freed of air, kept under a layer of paraffin oil, and should be used within 4 hours after having been rendered air-free.

Caprylic Alcohol.—This is used to prevent foaming.

Procedure.

The analysis consists of the following steps.

1. The gases, CO_2, O_2, CO, and N_2 are extracted from the blood sample in the chamber of the Van Slyke-Neill apparatus.

2. CO_2 is absorbed by the addition of N NaOH.

3. The mixture of residual gases, O_2, CO, and N_2 is transferred to the Hempel pipette containing alkaline pyrogallate, which absorbs the oxygen.

4. The blood is removed from the extraction chamber and replaced by air-free glycerol-salt solution.

5. The mixture of residual gases, CO and N_2, is returned to the chamber of the apparatus.

6. CO is absorbed by the addition of Winkler's reagent.

The details of the successive steps are given below. Since much of the technique has already been described in papers on other manometric analyses, it would be advantageous for the reader not already familiar with the manometric apparatus to consult previous papers referred to (1–4) for more complete explanations of general details and precautions.

The directions below apply when 2 cc. samples are used. The procedure for 1 cc. samples is given in a later section.

1. Extraction of Gases from Blood Sample.—From the cup of the Van Slyke-Neill apparatus, 2 drops of caprylic alcohol are admitted into the extraction chamber, followed by 8 cc. of the acid ferricyanide reagent. The stop-cock is sealed with mercury, and the chamber is evacuated and shaken for 2 minutes. The extracted air is ejected ((4) p. 428) and 4 cc. of the air-free reagent are allowed to run up into the cup. The blood sample is delivered from a 2 cc. rubber-tipped, stop-cock pipette ((1) p. 531). Traces of blood remaining in the cup are washed into the chamber with 1 cc. of the reagent, and the stop-cock is sealed. The chamber is evacuated and shaken for 3 minutes to extract the blood gases.

2. Absorption of CO_2 with NaOH.—Mercury is readmitted to

the chamber until the level of the liquid above comes to within a few cc. of the 2.0 cc. mark. 2 cc. of air-free N NaOH are placed in the cup, of which 1 cc. is slowly admitted into the chamber ((1) p. 545). The stop-cock is sealed and p_1, representing the total pressure of the gases O₂, CO, and N₂, is observed with the solution level at the 2.0 cc. mark.[1]

3. Transfer of Gases to Hempel Pipette and Absorption of Oxygen.—The Hempel pipette ((4) p. 437) contains alkaline pyrogallate protected by a layer of oil in the upper bulb. A little of the solution is run out to clear the stop-cock a (Fig. 1) of any air that may be present, then the capillary limbs, l and r, are filled with mercury from the cup c above. 1 cc. of mercury is poured into the cup k of the Van Slyke-Neill apparatus, and all air is dislodged from the capillary leading down from the cup to the chamber.

The stop-cock of the manometric apparatus which admits mercury from the leveling bulb to the extraction chamber is opened, and the leveling bulb is raised to such a height, (this will have to be determined by the analyst) that the extracted gases will be compressed into a bubble at the top of the chamber at *slight* positive pressure. The stop-cock is closed, and the leveling bulb set at rest in the uppermost ring, above the chamber.

The free end of the Hempel pipette, with mercury flowing through l from the cup c above, is thrust firmly down into the cup k so that the rubber tip fits snugly. Stop-cock a is opened in the position indicated (Fig. 1). Stop-cock b is then opened. At this point, if the internal pressure of the gas bubble has been correctly fixed, a small amount of the gas should run into the capillary limb of the Hempel pipette under its own pressure. The rest of the gas, followed by the blood solution, is forced up into the Hempel pipette by admitting mercury slowly from the leveling bulb into the extraction chamber. As soon as the blood solution has passed slightly beyond the stop-cock a, the latter is turned in a clockwise

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[1] If the precipitate formed by the interaction of the blood with the acid reagent obscures the meniscus, gentle movement of the chamber by hand will facilitate the solution of the proteins in the added alkali. Reading p_1 may then be taken over a clear solution. When dealing with darkly colored solutions, a source of light placed in back of the chamber has been found to be of great help in the adjustment of the meniscus to the volume mark. The light should only be used momentarily at the time of adjustment, so that no increase in temperature of the jacket and chamber may occur.

direction to the closed position shown in Fig. 1, position a, and the Hempel pipette is withdrawn.[2]

The free arm l is cleared of blood solution by the admission of mercury from cup c, and the capillary r, by continued turning of a in a clockwise direction, is likewise cleared of blood solution and gas. The pipette is set aside for the absorption of oxygen. Occasional gentle movement of the gas bubble to and fro, or in a horizontal rotatory manner facilitates the absorption, which is complete in 3 to 4 minutes.
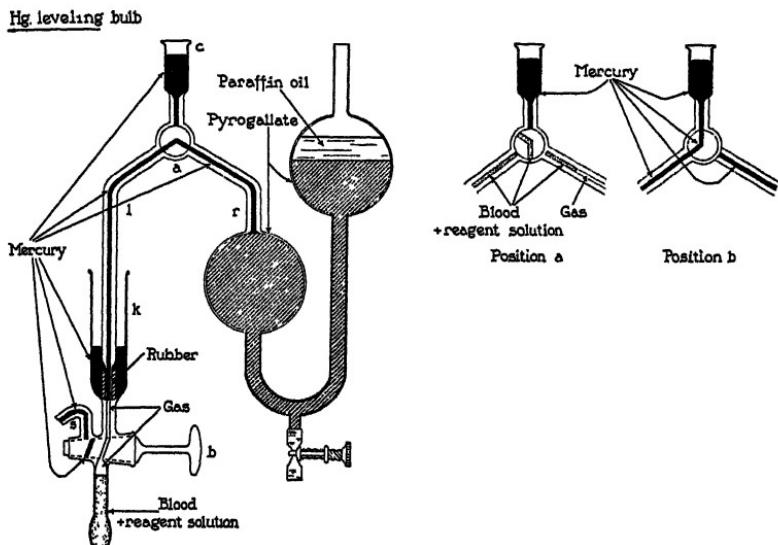
FIG. 1. Arrangement of apparatus for different stages of transfer of gas between extraction chamber and Hempel pipette.

4. Replacement of Blood Solution by Air-Free Glycerol-Salt Solution in Extraction Chamber.—In the meanwhile, the blood solution is removed from the chamber, which is then flushed two or three times with water. 5 cc. of glycerol-salt solution are then admitted into the apparatus and rendered air-free by shaking the evacuated

[2] In order to minimize the contamination of the absorbent by the blood solution, which precipitates in the pyrogallate and may even serve to trap gas, it is important to allow as little blood solution to pass into the capillary limb r as possible.

chamber for 2 minutes. The extracted air is expelled, and 1.5 cc. of the glycerol-salt solution are admitted into the cup k, 3.5 cc. remaining in the chamber.

5. Transfer of CO and N₂ from Hempel Pipette to Extraction Chamber.—1 cc. of mercury is poured into the cup k, stop-cock a is turned counter clockwise, and the Hempel pipette is placed in position while mercury is flowing from cup c into cup k (Fig. 1, position b). Stop-cock a is then turned counter clockwise to the position shown in Fig. 1. The mercury leveling bulb is placed in its lower position and stop-cock b is opened. The stop-cock connecting leveling bulb and extraction chamber is carefully opened, and, by withdrawal of mercury from the bottom of the chamber, the gas bubble from the Hempel pipette is slowly drawn into the top of the chamber. The minimum possible amount of pyrogallate solution is allowed to flow past the stop-cock a, which is then again turned back to the position indicated in Fig. 1, position b. By careful manipulation, with alternate opening and closing of the stop-cock a, the last portion of gas (and a slight amount of pyrogallate) is completely driven down, followed by mercury from cup c into the chamber, which is then sealed through stop-cock b.

The glycerol-salt solution level is lowered slightly below, then allowed to come to rest at the 2.0 cc. mark where a reading is taken. Due to the slow drainage of the viscous solution, two or three successive readings may be necessary to obtain the constant final reading, to be denoted as p_2. When CO is not present in too great amount, greater accuracy in its measurement is gained by obtaining a new base line reading, denoted by p'_2, at the 0.5 cc. mark. In this work, most of the CO pressure differences have been read at the 0.5 cc. mark.

6. Absorption of CO by Winkler's Solution.—6 cc. of the air-free Winkler's reagent are placed in cup k. Of this, 5 cc. are slowly admitted into the chamber at slight negative pressure (leveling bulb at height corresponding to the bottom of the chamber ((3) p. 581)). Due to the trace of pyrogallate which has followed the gas from the Hempel pipette into the chamber, the introduction of the first few drops of CO absorbent causes a precipitate to form. This, however, upon further addition of the reagent, drops to the bottom of the liquid, leaving the top with a clear meniscus. Absorp-

tion of CO is complete in 2 minutes. The solution is gently lowered to the 0.5 or 2.0 cc. mark, and the pressure p_3 is observed (see Van Slyke and Robscheit-Robbins (2) for precautions).

Determination of Corrections c_1 and c_2.—Before calculating the amounts of oxygen and carbon monoxide from the pressure differences $p_1 - p_2$ and $p'_2 - p_3$ (or $p_2 - p_3$), respectively, it is necessary to apply a correction in each case. Such corrections take into account whatever amounts of air may have been introduced with the reagent and not extracted the first time, and differences in pressure readings which result when either the *vapor tension* or the *volume* of the liquid above the mercury is altered.

To obtain the first correction c_1, the procedure previously outlined is followed. 2 drops of octyl alcohol and 8 cc. of the acidified ferricyanide reagent are extracted for 2 minutes, the extracted air is ejected, and 4 cc. of the solution are admitted into the cup k. 3 cc. are readmitted into the chamber and the extraction is repeated for 3 minutes. 1 cc. of air-free N NaOH is added and the reading p_2 made at the 2.0 cc. mark.

The chamber is cleaned and 5 cc. of glycerol-salt solution are extracted for 2 minutes. Following the ejection of extracted air, 3.5 cc. of the solution are left in the chamber. Reading p_2 is then made at the 2.0 cc. mark.

The difference in pressure $p_1 - p_2 = c_1$ and represents the correction to be applied to the $p_1 - p_2$ difference obtained in the analysis of the blood. In this work, the reading at 2.0 cc., over 3.5 cc. of glycerol-salt solution, has been found to be consistently between 1 and 2 mm. lower than the same reading over 8 cc. of the ferricyanide-NaOH mixture. This correction is largely the algebraic sum of two factors, namely, the *increase* in p_2 over p_1 due to the decrease in volume from 8 cc. to 3.5 cc., and the *decrease* in p_2 from p_1 due to the lower vapor tension of the glycerol-salt solution as compared with the tension of the ferricyanide-NaOH mixture.

To obtain the second correction c_2, a reading p'_2 at the 0.5 cc. mark is taken with the same 3.5 cc. of glycerol-salt solution, immediately after the 2.0 cc. reading. After the addition of 5 cc. of air-free Winkler's reagent, the reading p_3 is taken at the 0.5 cc. mark. When readings for CO are made at the 2.0 cc. mark (which, however, will occur only in analyses of blood of high CO content), the p'_2 reading is omitted, and p_3 is read at 2.0 cc., as in the analysis.

The difference in pressure

$$p'_2 - p_3 \text{ (or } p_2 - p_3) = c_2$$

and represents the correction to be applied to the $p'_2 - p_3$ (or $p_2 - p_3$) difference obtained in the analysis of the blood. At the 0.5 cc. mark the reading over 5 cc. of Winkler's reagent has been found to range from 3 to 5 mg. lower than the same reading over 3.5 cc. of glycerol-salt solution. Here also, the correction is the resultant of two factors; namely, the *decrease* in p_3 from p'_2 due to increase in volume from 3.5 to 5.0 cc., and the *increase* in p_3 over p'_2 due to the higher vapor tension of the glycerol-salt-Winkler's solution mixture as compared with the tension of the glycerol-salt solution alone.

The analyst should determine these two corrections for each day's analyses. If a new lot of any reagent is introduced during a series of analyses, the c corrections are redetermined.

Calculations.

The pressure of O_2 gas from the sample analyzed is calculated as

$$p_{O_2} = p_1 - p_2 - c_1$$

where the O_2 content in terms of volumes per cent or millimols per liter is calculated as

$$O_2 \text{ content} = p_{O_2} \times \text{factor.}$$

The values of the appropriate factor are taken from Column 9, Table II or Table III, of Van Slyke and Neill's paper (1) for a sample of 2 cc., $S = 7$ cc., $a = 2.0$ cc., and $i = 1.0$.

The pressure of CO gas from the sample analyzed is calculated as

$$p_{CO} = p'_2 - p_3 - c_2$$

where the CO content in terms of volumes per cent or millimols per liter is calculated as

$$CO \text{ content} = p_{CO} \times \text{factor.}$$

The values of the appropriate factor are taken from Column 8 of the same tables, Tables II and III (1), for a sample of 2 cc.,

$S = 7$ cc., $a = 0.5$ cc., and $i = 1.0$.   In some instances, when CO is present in large amounts, it may be necessary to take these readings (p_2 and p_3) at the 2.0 cc. mark, in which case the factors used will be the same as those for oxygen given above.

Estimation of O_2 and CO with 1 Cc. Blood Sample.

When the blood is about half saturated with O_2 and half with CO, or when it is necessary to economize in the amount of material used, one may obtain accurate results with blood samples of 1 cc. When one or both gases are present in small amount, there is a decrease in the percentage accuracy with which the less abundant gas is determined, since the absolute error (about 0.001 cc. of gas) remains constant.   Whether the resultant error is relatively too great to permit use of the 1 cc. sample depends upon the purpose of the analysis.   The amounts of reagents required are less than when 2 cc. samples are used, *all* readings are made at 0.5 cc., and the c corrections are different.

Briefly, the changes from the technique described for 2 cc. samples are as follows.   Instead of 8 cc. of acid ferricyanide, only 5.5 cc. are rendered air-free.   Of this, 4 cc. are run up into the cup k, and the 1 cc. sample is introduced into the chamber.   This is then followed by 1 cc. of the reagent, thus making 3.5 cc. of liquid to be shaken.   Of the N NaOH used to absorb the CO_2, only 0.5 cc. is introduced into the chamber.   Reading p_1 is made at 0.5 cc. After the return of the gas from the Hempel pipette, reading p_2 is taken over 3.5 cc. of air-free glycerol-salt solution at the 0.5 cc. mark.   For the CO absorption, 3.5 cc. of air-free Winkler's solution are introduced into the cup, of which 2.5 cc. are used for absorption.   Reading p_3 is also taken at the 0.5 cc. mark.   The c_1 and c_2 corrections are determined in the manner described in the previous section, the appropriate amounts of reagents being used.

The gas contents are calculated from

$$O_2 \text{ content} = (p_1 - p_2 - c_1) \times \text{factor}$$

and

$$CO \text{ content} = (p_2 - p_3 - c_2) \times \text{factor}$$

where the factor to be applied is obtained from Column 6, Table II of Van Slyke and Neill's tables of factors (1) for sample = 1 cc., $S = 3.5$ cc., $a = 0.5$ cc., and $i = 1.0$.

Determination of Blood O_2 or CO Alone.

Obviously, the procedure described with 1 or 2 cc. blood samples may be used when either O_2 or CO content alone is the aim of the analyst. While this method offers no advantage over the original Van Slyke-Neill technique for blood O_2, for CO it is less subject to the possible errors pointed out by those authors ((1) pp. 563 and 564).

When CO content alone is desired, the use of N NaOH is omitted. The gases liberated by the acid ferricyanide are transferred directly to the Hempel pipette, where CO_2 and O_2 are absorbed by the pyrogallol solution. The base line pressure reading p_1, with 3.5 cc. of air-free glycerol-salt solution, is made at either the 0.5 or 2.0 cc. mark. The final reading p_2, after the CO absorption by Winkler's reagent, is taken at the same volume as that at which p_1 was read. The appropriate c corrections in each of the above cases are determined as before.

EXPERIMENTAL.

The present method has been rigorously tested and compared with four other different techniques for the analysis of oxygen or carbon monoxide or a mixture of both, in blood. The results given in the following serve to indicate both the relative accuracy of the various methods, and the absolute accuracy of the newly modified procedure. The data presented are representative but not selected results. Of all the analyses done in preliminary tests of the method less than 10 per cent would have to be discarded because of inaccuracies outside the limit of experimental error.

Analysis of Blood for O_2 Content Only.

In order to test the accuracy of the gas bubble transfer from the extraction chamber to the Hempel pipette and back again, results of blood oxygen content analyses by the new technique were compared with values obtained by the method of Van Slyke and Neill. Table I is indicative of the good agreement obtained.

Analysis of Blood for CO Content Only.

For the purpose of comparing results with the Van Slyke-Robscheit-Robbins (2) technique, the experiments grouped in Table II were performed. They indicate good agreement. Even

when CO is present to the extent of only 4.5 volumes per cent, the comparison of results from 2 cc. samples used in the new technique, with results from 5 cc. samples by the Van Slyke-Rob-

TABLE I.

Results of Analyses of Blood for Oxygen Content Only.

Experiment No.	Volumes per cent O_2 by method of:			
	Van Slyke-Neill.		Authors.	
		Average.		Average.
1	20.40	20.40	20.34	
			20.32	20.33
2	24.40		24.28	
	24.35	24.38	24.34	24.31
3	20.19		20.25	20.25
	20.28			
	20.33	20.27		
4	21.99		21.97	
	21.93	21.96	21.95	21.96

TABLE II.

Results of Analyses of Blood for CO Content Only.

Experiment No.	Volumes per cent CO by method of:			
	Van Slyke-Robscheit-Robbins.		Authors.	
		Average.		Average.
1	8.12		8.07	
	8.36	8.24	8.07	8.07
2	11.57		11.56	
	11.61	11.59	11.56	11.56
3	4.66		4.75	
	4.62	4.64	4.72	4.73

scheit-Robbins method, shows no appreciable difference between the two sets of determinations.

The reliability of the method for CO absorption was tested in

another way. A portion of fresh ox blood was placed in the double tonometer system used in this laboratory (5). The system was evacuated and refilled with hydrogen three times, then equilibrated by rotation for 20 minutes at room temperature. The process of blood reduction and elimination of oxygen was repeated. Following this, a calculated amount of pure CO gas made from formic acid was added to the system. A low tension of 10 or 12 mm. was sufficient to saturate the blood thoroughly with CO while the amount physically dissolved at this pressure was so small (0.02 to 0.03 volume per cent) as to be negligible. The blood and gas phase were again allowed to come to equilibrium, after which the blood was analyzed for CO according to directions given in preceding sections.

Another portion of blood was evacuated and equilibrated the same number of times as the first, except that air was used as the gas phase. The O₂ capacity was finally estimated by analyzing for oxygen according to Van Slyke and Neill (1) and subtracting the physically dissolved O₂ according to the equation

$$(1) \quad \text{Dissolved O}_2 \text{ (vol. per cent)} = \frac{B - W}{760} \times 20.9 \times \alpha_{O_2} \times 0.84$$

where $B - W$ represents the barometric pressure minus the water vapor tension at the temperature of saturation and α_{O_2} is the Bunsen solubility coefficient at the same temperature, for oxygen in water. The numerical constants 20.9 and 0.84 are respectively the percentage of oxygen in air, and the approximate water content of blood.

The combining capacity of the blood for O₂ and CO should be the same. Table III shows the results of two such experiments. In Experiment 1, it may be added, further analyses indicated an oxygen content of 0.25 volume per cent, incomplete reduction of the sample saturated with CO thus accounting for the slightly lower CO results.

Finally, the CO content of a sample of blood thoroughly saturated with that gas was confirmed in still another way. The blood was equilibrated with an atmosphere of almost pure CO, then put aside in a closed vessel over mercury. 2 cc. samples were withdrawn for analysis according to the method described in this paper. After analysis, the amount of CO *unextracted* in the course

of the analysis was calculated by multiplying the *total* CO by the appropriate factor for unextracted gas obtained from the equations developed by Van Slyke and Stadie (6). At equilibrium,

TABLE III.

Comparison of O_2 and CO Blood Capacities.

Experiment No.	Volumes per cent gas by method of:					
	Van Slyke-Neill.			Authors.		
	O_2			CO		
		Average.				Average.
1	22.36			22.12		
	22.32	22.34		22.18		22.15
2	19.67			19.71		
	19.81	19.74		19.71		19.71

TABLE IV.

Determination of CO Extracted from Blood in Van Slyke-Neill Apparatus.

Volumes per cent CO.

Total by authors' method.		Calculated.		Extracted, analysis by I^2O^5 method.	
	Average.	Unextracted.	Extracted.		Average.
22.68				22.61	
22.53	22.60	0.09	22.51	22.93	22.77

the ratio of unextracted gas to the total gas present is defined by the equation

$$(2) \qquad \frac{\dfrac{A\,\alpha'}{A-S}}{1+\dfrac{A\,\alpha'}{A-S}}$$

where A = volume of gas phase, S = volume of liquid phase, and α' = the Ostwald solubility coefficient at the prevailing temperature. To determine the accuracy of the results, several other samples were extracted in the evacuated chamber of the Van Slyke

O₂ and CO in Blood

apparatus. In each case, after extraction, the stop-cock b (see Fig. 1) of the chamber was opened to dilute the extracted CO with outside air. The gas in the chamber was then completely transferred through the capillary side arm S of the apparatus through stop-cock b, by displacement with mercury, into an 800 cc. partially evacuated tonometer. Air was admitted into the tonometer to atmospheric pressure. Following this, the gas was displaced by glycerol-salt solution and passed over hot I_2O_5. The iodine liberated by the CO was absorbed in KI solution and

TABLE V.

Comparison of Results of Combined Analysis of Blood for Both O_2 and CO by Method of Van Slyke and Robscheit-Robbins and by That of the Authors.

Experiment No.	Volumes per cent gas by method of:							
	Van Slyke-Robscheit-Robbins.				Authors.			
	O₂		CO		O₂		CO	
		Average.		Average.		Average.		Average.
1	11.22	11.22	12.14	12.14	11.37		12.02	
					11.27	11.32	11.94	11.98
2	10.78		11.22		10.54		11.42	
	10.74	10.76	11.45	11.34	10.56	10.55	11.32	11.37
3	5.71	5.71	6.21		5.61		6.32	
			6.16	6.19	5.63	5.62	6.36	6.34
4	15.40		5.42		15.56		5.46	
	15.54	15.47	5.46	5.44	15.47	15.51	5.48	5.47
5	5.85		6.05		5.65		6.26	
	5.74	5.80	6.20	6.13	5.56	5.60	6.31	6.29

then titrated with sodium thiosulfate. More complete details as to the procedure employed will be given in a later publication from this laboratory. Table IV shows that CO, thus determined by an entirely independent method of measurement, agrees very well with the results obtained by the manometric technique.

Combined Analysis of Blood for O₂ and CO Content.

The accuracy of the procedure having been tested against other methods with respect to one or the other of the two gases, experiments were performed for the purpose of confirming results by the

combined technique. The first of several series of such experiments is recorded in Table V, where comparative results with respect to the Van Slyke-Robscheit-Robbins method are given. In view of the fact that the latter method was not designed for oxygen analysis, and its use for that purpose introduces several

TABLE VI.

Comparison of Results of Combined Analyses of Blood for Both O_2 and CO by Several Different Methods.

Experiment No.	Blood sample.	Content.	Volumes per cent gas by method of:						
			Van Slyke-Neill.	Van Slyke-Robscheit-Robbins.		Authors.		Calculated.*	
			O_2	O_2	CO	O_2	CO	O_2	CO
1	A	O_2 + CO		0.41	18.98				
				0.32	18.97				
	B	O_2	18.97						
			19.08						
	C	1 A : 1 B†					9.22		
							9.38	9.69	9.49
2	A	O_2 + CO		0.26	22.64				
				0.20	22.69				
	B	O_2	22.64	·					
			22.50						
	C	1 A : 1 B†				11.37	11.39		
						11.32	11.58	11.40	11.33
3	A	O_2	23.79						
			23.71						
	B	CO							
	C	1 A : 1 B†		11.85	12.54	11.86	12.55		
				11.93	12.55	11.89	12.55	11.88	

* O_2 values calculated from Van Slyke-Neill and Van Slyke-Robscheit-Robbins analyses. CO values calculated from Van Slyke-Robscheit-Robbins analyses.

† 1 part A + 1 part B.

extra steps in the published procedure, the agreement in values is quite satisfactory.[3]

[3] The additional steps involved in analyzing blood for oxygen by the Van Slyke-Robscheit-Robbins method were the following. After the extraction of the blood gases, 2 cc. of air-free N NaOH were added to absorb

Table VI gives comparative results, analyzed and calculated, for experiments with mixtures of ox blood saturated with CO and with O_2, the bloods having been analyzed for CO or O_2 before and after the mixture was prepared. From the preliminary analyses by the Van Slyke-Neill and Van Slyke-Robscheit-Robbins methods, values of O_2 and CO for the mixture were calculated. The agreement of the analytical data with the calculated values is as good as could be expected considering the number of steps involved in preparing the final mixtures.

The most rigorous test of the accuracy of results by the combined method is that based on the principle of the identity of the oxygen- and carbon monoxide-combining power of a given sample of blood. When blood is equilibrated with an atmosphere containing either O_2 or CO at a tension sufficient to have all of the available hemoglobin combined with gas, the amount of O_2 and CO combined in either case will be identical. The amounts of *dissolved* O_2 or CO will depend on the tension of the respective gases, as indicated by Equation 1 where CO may be substituted for O_2. As shown by Sendroy, Liu, and Van Slyke (7) the tension of CO required to saturate blood with that gas will be but $\frac{1}{210}$ part of the tension of O_2 required to combine all the blood hemoglobin with oxygen. In the one case, therefore, one may have the blood fully combined with CO, with a negligible amount of dissolved CO present, while in the other, when the blood is fully combined with O_2, a correction must be made for dissolved gas.

Table VII indicates the results obtained by analysis of mixtures of blood, the amounts of O_2 or CO in which could be calculated from O_2 or CO capacity data. Thus, for instance, in Experiment 1 (Table VII), a certain portion of blood (Sample A) was saturated with air, and analyzed for O_2 as in the regular O_2 capacity method

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CO_2, and the blood solution was removed from the Harington-Van Slyke chamber through the lower stop-cock. The chamber was washed once with air-free glycerol-salt solution. This was rejected and then replaced by another 5 cc. over which the reading at the 2.0 cc. mark was taken. The difference between this reading and the subsequent one following the absorption of oxygen by pyrogallate gave the pressure due to O_2. A new table of factors similar to that given by Van Slyke and Robscheit-Robbins was calculated for use in oxygen analyses.

TABLE VII.

Comparison of Results of Combined Analyses of Blood for CO and O_2 Compared with Values Calculated from O_2 Capacity Results.

xperiment No.	Blood sample.	Volumes per cent gas by method of:			Calculated.*	
		Van Slyke-Neill.	Authors.			
		O_2	O_2	CO	O_2	CO
1	A, saturated with air.	20.36t 19.84c				
	B, saturated with CO at 10 mm.			19.93		19.84
	C = 3 parts A + 1 part B.		15.06	5.05	15.25	4.96
2	A, saturated with air.	20.34t 19.82c				
	B, saturated with CO at 10 mm.			19.85		19.82
	C = 1 part A + 1 part B.		10.19	9.97	10.17	9.92
3	A, saturated with air.	20.65t 20.16c				
	B, saturated with CO at 10 mm.					20.16
	C = 5 parts A + 1 part B.		17.20	3.43	17.21	3.36
	D = 5 parts A + 2 parts B.		14.79	5.75	14.75	5.76
4	A, saturated with air.	21.76t 21.26c				
	B, saturated with CO at 10 mm.					21.26
	C = 15 parts A + 2 parts B.		19.10	2.58	19.20	2.50
	D = 5 parts A + 1 part B.		17.90	3.63	18.13	3.55
5	A, saturated with air.	22.59t 22.09c				
	B, saturated with air containing CO.		0.33	21.69		
	C = 1 part A + 5 parts B.		4.21	17.96	4.05	18.06
6	A, saturated with air.	19.45t 18.98c				
	B, saturated with CO at 10 mm.					18.98
	C = 1 part A + 2 parts B.		6.59	12.57	6.49	12.65
	D = 1 part A + 5 parts B.		3.26	15.59	3.24	15.81

TABLE VII—*Concluded.*

Experiment No.	Blood sample.	Volumes per cent gas by method of:			Calculated.*	
		Van Slyke-Hiller.	Authors.			
		CO	O_2	CO	O_2	CO
7	A, saturated with air.				22.07t 21.57c	
	B, saturated with CO at 10 mm.	21.57t				
	C = 2 parts A + 1 part B.		14.54	7.13	14.71	7.19

t = total gas.   c = combined gas.

* O_2 values calculated from total O_2 according to Van Slyke-Neill method .   CO values calculated from combined O_2 values.

of Van Slyke and Neill.   The total O_2, by analysis, was 20.36 volumes per cent.   The calculated dissolved O_2 was 0.52 volumes per cent, thus making the O_2-combining power of the sample equal to 19.84 volumes per cent.   Hence, the CO-combining power of the same blood, when exposed to an atmosphere containing CO at sufficient tension, should be identical with this value.

Accordingly, a second portion of blood (Sample B, Experiment 1) was saturated with an atmosphere containing enough CO to combine completely with the amount of blood present and to have in excess an amount which would make the CO tension at equilibrium 10 mm. of mercury.   The dissolved CO was therefore negligible.

The saturation of the blood in each case was made at room temperature and repeated to make three saturations in all, according to the technique of Austin *et al.* (5).   Hence, the concentration of the blood, due to the several evacuations of the tonometers, was the same in each case.

Blood (Sample B, Experiment 1) was analyzed for CO according to the new technique and the results (19.93 volumes per cent) checked well with the calculated value (19.84 volumes per cent) obtained from the Van Slyke-Neill analysis for O_2 capacity.

Definite volumes of blood (Samples A and B) were then accurately measured out and mixed, 3 parts by volume of Sample A to

one of Sample B, and the analyses carried out by the combined technique.   Again the results were compared with the calculated values.

In Table VII, there has been included, for convenience in presentation, an experiment somewhat different from the preceding ones.   In Experiment 7 the procedure employed was the reverse of that in the others, in that the *CO capacity*, as estimated by the

TABLE VIII.

Comparison of Combined Analyses of Blood for O_2 and CO with 1 Cc. and 2 Cc. Samples.

Experiment No.	Volumes per cent.							
	O_2				CO			
	1 cc. sample.		2 cc. sample.		1 cc. sample.		2 cc. sample.	
		Average.		Average.		Average.		Average.
1	10.03	10.03	10.11	10.11	11.09	11.09	11.01	11.01
2	10.80		11.01		11.77		11.71	
	10.71		10.95	10.98	11.70		11.73	11.72
	10.91	10.81			11.65	11.71		
3	16.05		16.01		6.17		6.18	
	16.16	16.10	15.96	15.99	6.15	6.16	6.19	6.18
4	11.13		10.97		11.56		11.76	
	11.00	11.06	10.92	10.95	11.62	11.59	11.78	11.77
5	19.07		18.89		3.18		3.12	
	18.72		18.87	18.88	3.20		3.15	3.14
	18.88	18.89			3.18	3.19		

method of Van Slyke and Hiller (8), was used as the basis of calculation of O_2 values.   Actually, blood Samples A and B were equilibrated as before, with air and with a low tension CO + H_2 mixture.   The blood mixture (Sample C) was prepared and kept over mercury.   After analysis for O_2 and CO by the combined technique, samples of the same mixture were employed for the estimation of the CO capacity within the apparatus.   Experiment 7 thus constitutes another independent confirmation of the values

given by the modified technique. The agreement of calculated and analyzed values in this table is within the limit of error to be expected in the preparation by volume of blood mixtures such as these.

Analysis of 1 Cc. Samples.

In order to determine the relative accuracy of results obtained by reducing the size of the blood sample to 1 cc., the results given in Table VIII were obtained. Apparently, for the mixtures here used, 1 cc. and 2 cc. samples give results agreeing within the limit of error of the method.

SUMMARY.

An improved technique is described for the determination of oxygen and carbon monoxide in a single blood sample by the use of the Van Slyke-Neill manometric apparatus.

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ON THE OPTICAL ROTATION OF *l*-CYSTINE.

DETERMINATION OF ITS VALUE FOR THE SODIUM AND MERCURY LINES AND OF THE TEMPERATURE FACTOR.

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The only practical quantitative criterion for the degree of purity of *l*-cystine is its optical rotation. The importance of a well established and well defined numerical value is obvious in view of the biological and chemical interest attached to this substance.

The present work deals with the purification of *l*-cystine, by fractionated precipitation, and with rotatory determinations for the Hg and the D lines between 20 and 30°. The literature contains no data on the $[\alpha]_{Hg}$ values of *l*-cystine. The $[\alpha]_D$ values presented which, as well as the $[\alpha]_{Hg}$ values, were determined under the standard conditions established by Andrews (1), differ substantially from his values for the rotation and for the temperature coefficient.

EXPERIMENTAL.

For the purification of crude cystine obtained from hair and wool and previously freed from tyrosine and partly decolorized, use was made of the observations of various authors (2, 3) that the inactive and dextrorotatory modifications are more soluble in water than the *l*-compound. The material was repeatedly boiled out with large volumes of water adjusted to pH 3 to 4 by means of a few drops of HCl, treated with charcoal in HCl solution, reprecipitated by alkali, and filtered immediately. 20 per cent of the starting material was thus removed; the microscopic examination of the single evaporation residues showed that it consisted largely of fine white needles, in addition to colored impurities present in the first extractions.

A sample of the purified cystine was dried to constant weight in presence of P_2O_5 *in vacuo* at 111° and gave the following rotations in 1.02 N HCl. (This acid was used as the solvent in all the following determinations.)

$$[\alpha]_{Hg}^{28} = \frac{-4.894° \times 25 \text{ cc.}}{2 \times 0.261 \text{ gm.}} = -234.4°.$$

$$[\alpha]_{D}^{28} = \frac{-4.142° \times 25 \text{ cc.}}{2 \times 0.261 \text{ gm.}} = -198.4°.$$

40 gm. of this material now were extracted four times with water, without pH adjustment, and a fifth time with slightly acid water (pH 3 to 4), 1500 cc. of H_2O being used each time. About 5 per cent was removed by these extractions, and the last evaporation residues seemed to consist wholly of the hexagonal crystals of *l*-cystine. The purified cystine was dissolved in 600 cc. of N HCl, precipitated at pH 4 by adding N NaOH and N NaOOCCH$_3$ (total volume of solution 1500 cc.), filtered after standing a short time, and washed with water, alcohol, and ether, yielding 35 gm. of material.

$$[\alpha]_{Hg}^{29.36} = \frac{-4.7445° \times 25.505 \text{ gm.}}{2 \times 0.2504 \text{ gm.} \times 1.017} = -237.59°.$$

$$[\alpha]_{D}^{28-28.5} = \frac{-4.077° \times 25 \text{ cc.}}{2 \times 0.2500 \text{ gm.}} = -203.85°.$$

Since on previous attempts, we never succeeded in increasing the maximum rotation more than 1 or 2 degrees beyond these figures, either by continued extraction with water or by reprecipitations with charcoal treatments, a different procedure was chosen to complete the isolation of pure *l*-cystine; *viz.*, fractionated precipitation at hydrogen ion concentrations outside the isoelectric range (4):

3 gm. of the material described above were dissolved in 50 cc. of N HCl (at least 50 per cent more than the stoichiometrically required amount was always found necessary to dissolve the cystine). After filtering and diluting with 300 cc. of H_2O, N NaOH was added until the pH of the solution was about 1.1. Crystallization began immediately; after allowing to stand overnight the precipitate was filtered, and washed with alcohol and ether. By further addition of NaOH to the filtrate (now 500 cc.)

the pH was brought to 1.6 and the crystallization, which was very slow this time, produced a small volume of heavy, large, hexagonal crystals. They were filtered off and, after removing the filtrate, washed with water, alcohol, and ether as before. The filtrate then was brought to pH 4.7, the crystallized precipitate that formed immediately was filtered off and washed as usual.

A summary of fractionation and optical determinations is as follows:

Fraction I. pH 1.1, 40 per cent yield, 0.2500 gm. cystine in 25.518 gm. solution.

Fraction II. pH 1.6, 27 per cent yield, 0.2510 gm. cystine in 25.4935 gm. solution.

Fraction III. pH 4.7, 24 per cent yield, 0.2502 gm. cystine in 25.5115 gm. solution.

The specific gravities used are based on the determinations reported later.

Fraction No.	t (average) °C.	$-°\alpha$ Hg (average).	$-°[\alpha]_{Hg}^{t}$
I	29.20	4.801	240.93
II	29.86	4.801	239.77
II	29.32	4.826	241.00
III	29.45	4.757	238.48
III	29.31	4.754	238.31
III	30.48	4.709	236.14

Since Fractions I and II seemed to show no appreciable differences, a solution of Fraction I was used to study in detail the relation between D and Hg rotations and the influence of temperature (see Tables I and II).

Method of Optical Determinations.

A Landolt precision polarimeter with 0.01° vernier divisions made by Schmidt and Haensch was used. The light source for the D line (5892.5 Å) was a Bunsen burner the flame of which was fed by air pressure with a spray of a 20 per cent NaCl solution by means of a glass aspirator and a metal hood attachment on the burner.[1] The light from this lamp passed through a 30 mm.

[1] This arrangement, which is furnished by the Central Scientific Company, Chicago, can be recommended.

layer of 3 per cent $K_2Cr_2O_7$ solution inserted in the front end of the polariscope. For the Hg line (5461 Å) a Lab-Arc Hg lamp[2] with two Corning glass filter plates, "G-555-B.E. didymium, 5.13 mm. thick" and "G-34-y", was used. The instrument was standardized for the two lines used against a quartz control plate with a Bureau of Standards certificate. A half shadow angle of $3-3\frac{1}{2}°$ for the D line and of $1\frac{3}{4}°$ for the Hg line gave the most accurate readings. For each group of determinations a blank of the empty instrument was determined. For all of the following determina tions (as for some of the preceding ones) a Schmidt and Haensch 200 mm. tube (model No. 83) with water jacket and ground-in thermometer (10–32° in 0.1° divisions) was used. The thermometer was checked against a Bureau of Standards standard. After trying various constant temperature devices, an electric rheostat with water circulation[3] was found to furnish a simple and suitable arrangement in connection with a constant level water container fed directly from the cold water line. By varying the height of the water container and, if necessary, the rheostat adjustment, any desired temperature could quickly be obtained. When the water jacket of the tube was supplied in this manner the temperature in the tube as a rule did not vary more than 0.1–0.2° during the time required to take a group of about 10 single readings. At each temperature level a group of at least 8 readings was taken, the temperature being registered each time immediately after the polariscope adjustment. By averaging within each group the recorded temperatures and rotation angles, the figures tabulated below were obtained. They are corrected for a small constant deviation produced by the tube filled with the pure HCl.

The specific gravities were determined at three temperatures for a 25 cc. solution containing 0.250 gm. of cystine in 1.02 N HCl, by comparing the weight of the solution with an equal volume of freshly boiled water. The probable accuracy of these determinations is ±0.0002 corresponding to about ±0.04° [α]. The following values were found: 1.0190 for 20°, 1.0180 for 25°, and 1.0168 for 30°. By interpolation from these figures the specific gravities corresponding to the experimental temperatures were obtained.

The solution used on both the D and Hg series contained 0.2500

[2] Cooper Hewitt Electric Company.
[3] Central Scientific Company.

gm. of cystine (Fraction I, see above) in 1.02 N HCl, the weight of the solution being 25.518 gm.

The rotatory determinations are given in Tables I and II.

By dividing each series into two equal groups (of five and one-half and six and one-half members, respectively) and developing the equations of the straight lines from the formulæ

$$\Sigma'y = \Sigma'a - b\,\Sigma'x$$

and

$$\Sigma''y = \Sigma''a - b\,\Sigma''x$$

where Σx represents the sum of the $[\alpha]$ values found and Σy the sum of the corresponding temperatures, while Σ' and Σ'' refer to the sums of the first and second halves of the readings, respectively, we obtain the two equations

(1) $\qquad y = 128.18 - 0.48509\,x$ for the D series

and

(2) $\qquad y = 128.62 - 0.41305\,x$ for the Hg series

or, for the actual specific rotations

(3) $\qquad [\alpha]_D^t = 2.0615\,t° - 264.24°$

and

(4) $\qquad [\alpha]_{Hg}^t = 2.421\,t° - 311.40°$

In Column 5 of Tables I and II, the $[\alpha]$ values calculated from these equations are given and in Column 6 the deviations. From equations (1) and (2), it appears that the intercept on the y axis is practically the same for both series; and this points strongly to the fact that actually both rotations become zero at the same temperature. A calculation based on this assumption changes the figures merely to an insignificant extent and agrees with the experimental data just as well. Therefore, and in view of the fact that, as judged from the unusually large temperature factor, the optical rotation of the l-cystine cation presents a case quite out of the ordinary[4] we assume that the temperature curves of the D and Hg rotations are straight lines over the range investigated

[4] Cf. the interesting review on the determining factors of optical rotation and dispersion by Patterson (5).

D Series.

Average temperature.	Average rotation.	Specific gravity.	Specific rotation found.	Specific rotation calculated.	Deviation.	
(1)	(2)	(3)	(4)	(5)	(6)	
$t\,°C.$	$-°\alpha$	d_4^t	$-°[\alpha]_D^t$	$-°[\alpha]_D^t$		
30.25	4.025	1.0167	202.04	201.88	+0.16	
29.88	4.040	1.0168	202.77	202.645	+0.125	
29.65	4.042	1.0169	202.87	203.115		−0.245
29.16	4.074	1.0170	204.46	204.125	+0.335	
28.10	4.100	1.0173	205.70	206.31		−0.61
26.45	4.191	1.0177	210.18	209.71	+0.47	
24.91	4.242	1.0180	212.66	212.89		−0.23
24.35	4.277	1.0181	214.40	214.04	+0.36	
22.59	4.334	1.0185	217.19	217.67		−0.48
21.47	4.384	1.0187	219.63	219.98		−0.35
20.54	4.439	1.0189	222.36	221.895	+0.465	
					+1.915	−1.915
					+1.915	
					+3.830 ÷ 11 = ±0.35°	

TABLE II.
Hg Series.

Average temperature.	Average rotation.	Specific gravity.	Specific rotation found.	Specific rotation calculated.	Deviation.	
(1)	(2)	(3)	(4)	(5)	(6)	
$t\,°C.$	$-°\alpha$	d_4^t	$-°[\alpha]_{Hg}^t$	$-°[\alpha]_{Hg}^t$		
30.50	4.734	1.0167	237.65	237.56	+0.09	
30.11	4.754	1.0168	238.64	238.505	+0.135	
29.82	4.763	1.0169	239.06	239.205		−0.145
29.39	4.794	1.0170	240.59	240.245	+0.345	
28.90	4.808	1.0171	241.27	241.435		−0.165
28.01	4.848	1.0173	243.23	243.58		−0.35
26.17	4.948	1.0177	248.13	248.045	+0.085	
24.48	5.019	1.0181	251.59	252.135		−0.545
24.15	5.043	1.0182	252.79	252.935		−0.145
23.18	5.087	1.0184	254.94	255.28		−0.34
21.94	5.151	1.0186	258.08	258.285		−0.205
21.73	5.177	1.0187	259.38	258.795	+0.585	
20.32	5.248	1.0189	262.85	262.205	+0.645	
					+1.885	−1.895
					+1.895	
					+3.780 ÷ 13 = ±0.29°	

and that these straight lines would intersect at $[\alpha]_D = [\alpha]_{Hg} = 0°$ in accordance with the following equations.

(5) $$y_D = 128.19 - 0.48516\ x_D$$

and

(6) $$y_{Hg} = 128.19 - 0.41133\ x_{Hg}$$

or,

(7) $$[\alpha]_D^t = 2.061\ t° - 264.23°$$

and

(8) $$[\alpha]_{Hg}^t = 2.431\ t° - 311.65°$$

By dividing the average error between temperature and polariscope readings $\pm(0.06° - 0.003°\alpha_{Hg})$ is obtained as the average observation error.

The changes in specific rotation for 1° obtained from equations (7) and (8), $2.061°\ [\alpha]_D$ and $2.431°\ [\alpha]_{Hg}$, were now used for the correction of specific rotations to a common temperature basis. The figures obtained above on Fractions I, II, and III and their starting material, thus corrected to 29° give

Fraction I $[\alpha]_{Hg}^{29}$	Fraction II $[\alpha]_{Hg}^{29}$	Fraction III $[\alpha]_{Hg}^{29}$	Starting material $[\alpha]_{Hg}^{29}$
degrees	degrees	degrees	degrees
−241.42	−241.86	−239.57	−238.46
−241.15*	−241.78	−239.26	
		−239.74	
Average −241.29 ±0.14	−241.82 ±0.04	−239.52 ±0.18	

* From equation (8).

It appears now that the rotation of Fraction II is actually about 0.5° higher than that of Fraction I, while Fraction III rotates 1.8° lower than Fraction I and the best fraction (Fraction II) is about 3.4° above the starting material. The best separation, therefore, seems to take place between the fractions precipitated above and below a pH of about 2.

Consequently, Fractions I and II were combined and treated as follows: 1.55 gm. of cystine were suspended in 250 cc. of H_2O and dissolved by addition of 100 cc. of N HCl. To the filtered

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TABLE III.

Results of First Refractionation.

</div>

Fraction I A.   pH 1.7, 49 per cent yield.
    Solution 1.   0.2500 gm. cystine in 25.4635 gm. solution.
      "   2.   0.1530 "   "   " 15.801 "   "

Fraction I B.   pH 3.9, 39 per cent yield.
    Solution 1.   0.2508 gm. cystine in 25.457   gm. solution.
      "   2.   0.2501 "   "   " 25.4575 "   "
      "   3.   0.2252 "   "   " 25.659 "   "

Fraction.	Solution.	t (average) °C.	$-°\alpha$ Hg (average).	$-°[\alpha]_{Hg}^{29}$	Fraction.	Solution.	t (average) °C.	$-°\alpha$ Hg (average).	$-°[\alpha]_{Hg}^{29}$
I A	1	29.54	4.815	242.44	I B	1	29.35	4.816	241.20
		27.28	4.915	241.82			27.80	4.901	241.57
	2	29.49	4.739	241.83			27.03	4.933	241.25
		29.06	4.768	242.24		2	29.95	4.775	241.29
		26.29	4.872	242.26			28.13	4.868	241.44
		23.57	5.031	241.92			27.64	4.890	241.80
							25.50	4.990	241.00
						3	27.68	4.375	241.75
							28.87	4.311	241.13
							30.01	4.269	241.65
Average				−242.08 ±0.28°					−241.41 ±0.23°

<div align="center">

TABLE IV.

Results of Second Refractionation.

</div>

Fraction I AA.   pH 1.8, 60 per cent yield.
    Solution.   0.1218 gm. cystine in 13.7225 gm. solution.

t (average) °C.	$-°\alpha$Hg (average).	$-°[\alpha]_{Hg}^{29}$
24.28	4.675	241.74
24.79	4.553	241.69
26.32	4.488	241.89
28.05	4.406	241.65
30.25	4.313	241.97
Average..		−241.79 ±0.11°

solution N NaOH was added until the pH was 1.7. Crystallization appeared very slowly and was allowed to continue overnight. The precipitate was then filtered, washed with water, and after saving the filtrate, washed with alcohol and ether (Fraction IA). The filtrate (about 500 cc.) was brought to pH 3.9 by addition of N NaOH, the crystals being filtered off the next day and washed and dried as usual (Fraction IB, see Table III).

On comparing the figures given in Table III with the ones obtained on Fractions I and II, it is evident that the fractionation obtained is very similar to the preceding one. The reason why in the latter the first fraction had the slightly lower rotation may lie in the fact that the conditions of precipitation caused Fraction I to crystallize rapidly, thereby possibly carrying down traces of inactive cystine, while Fraction II crystallized very slowly and in well defined single hexagons. From these results it appears that, if the maximum rotation is still higher than the value obtained on Fraction I A, the tendency would be disclosed by a partial slow reprecipitation of Fraction I A at a pH lower than 2.

0.20 gm. of Fraction I A were dissolved in 3 cc. of N HCl and diluted with 96 cc. of H_2O. By addition of N NaOH, the pH was brought to about 1.8. Crystals began to form slowly; after 24 hours they were filtered, washed, and dried as usual (Fraction IAA, see Table IV).

The results on the various fractions are summarized in the accompanying diagram.
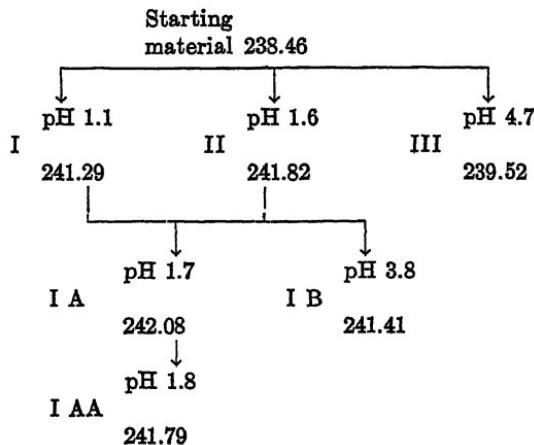
It appears that no further purification was obtained and that Fractions I, I A, and I AA gave the same rotation within the limits of experimental error.

Comparing the corresponding D figures with the one given by Andrews (1) and being aware of his work on the solubilities of the different optical modifications of cystine (3), we could not yet be satisfied as to the purity of our material. Consequently, a new preparation, starting with our cystine of $[\alpha]_{Hg}^{29} = -238.5°$, was made in the manner described by repeated fractionated precipitation at different pH values. We thus obtained a sample that gave the following rotations (corrected for 29°).

$$[\alpha]_{Hg}^{29} = -241.80°, -241.68°, -241.85°; \text{average}, -241.78° \pm 0.06°$$

This material was used in the following solubility determinations

TABLE V.
Solubility Determinations.

Bottle No.	Time of shaking.	Amount cystine present.	Cystine obtained in 50 cc. saturated solution.		Corresponding to gm. in 1000 cc.
	hrs.	*gm.*	*gm.*		
1	127	0.05	0.0066	0.0069	0.135 ±0.003
2	105	0.05	0.0067	0.0066	0.133 ±0.001
3	105	2.00	0.0068	0.0064	0.131 ±0.0025
			0.0065	0.0065	

Glass-stoppered bottles containing 300 cc. of H_2O and the amounts of cystine given in Table V were shaken on a machine for 4 and 5 days, respectively, at 24–27.5°. In the filtered solutions the amount of dissolved cystine then was determined by evaporating 50 cc. samples on the steam bath to a small volume, placing them in a vacuum desiccator over $CaCl_2$, and weighing after the residues were completely dry. The pH of the water used was, determined colorimetrically, 6.3 to 6.4 while the saturated solutions showed pH 6.4 to 6.5. The solubility figures obtained are somewhat higher than the minimum solubilities usually given[5] but they seem to be in fair agreement with the determinations of Sano (7) about solubility and hydrogen ion concentration. The

[5] For review see (3) and (6).

fact that no difference appears between the amounts dissolved from differently sized samples must, in view of the work of Andrews and De Beer (3), be considered as additional proof for the homogeneousness of our material.

Taking the grand average of the average determinations obtained on four different samples:

$-241.79°$ $\pm 0.11°$ (five single determinations)
$-241.82°$ $\pm 0.01°$ (two   "   ·   "   )
$-242.08°$ $\pm 0.28°$ (six   "   "   )
$-241.78°$ $\pm 0.06°$ (three   "   "   ) •

we find $[\alpha]_{Hg}^{20} = -241.87°$ $\pm 0.15°$ as the most probable expression for the specific rotation of l-cystine.

The standard value obtained on Fraction I was $[\alpha]_{Hg}^{20} = -241.15°$ or $0.72°$ less than the above value. The corrected equation (8) therefore would be

$$[\alpha]_{Hg}^{t} = 2.431 \ t° - 312.37°$$

and, herefrom calculated, the corrected equation (6)

$$y_{Hg} = 128.48 - 0.41133 \ x_{Hg}$$

and the corrected equation (5)

$$y_{D} = 128.48 - 0.48516 \ x_{D}$$

The best expressions for the specific rotation of l-cystine between 20 and 30° in a concentration of 1.0 gm. in 100 cc. of N HCl then are:

$$[\alpha]_{Hg}^{t} = (2.431 \ t - 312.37)° \pm 0.2°$$

and

$$[\alpha]_{D}^{t} = (2.061 \ t - 264.84)° \pm 0.2°$$

DISCUSSION.

In our work we were originally guided by the value $-215.5°$ established by Andrews (1) for the purpose of so standardizing conditions "that values . . . shall accurately indicate . . . the relative concentration of cystine." We followed the conditions established by Andrews for the concentration and encoun-

tered no difficulties. The numerical values, however, obtained under these conditions disagree with his in two respects. First, we obtained for $[\alpha]_D^{20}$ the value $-205.1 \pm 0.2°$ as compared with $-215.5°$, and second, for the average temperature coefficient between 20–30° we find $-2.06°[\alpha]_D$ for $-1°$ against $1.7°[\alpha]_D$ found by Andrews. Our experimental evidence, regarding the system used in purification and the method applied in the optical determinations, as well as our solubility data and the microscopic appearance of the crystals, indicate that we have been dealing with a practically pure preparation of *l*-cystine.

For our figures we find some support in the literature. Fischer and Suzuki (8), when establishing the disputed identity of cystine from hair and from cystine stones, found on careful purification "not only from tyrosin but also from racemic cystine:"

$$[\alpha]_D^{20} = \frac{-11.84° \times 12.2400 \text{ gm.}}{2 \times 0.3172 \text{ gm.} \times 1.029} = -221.9° \text{ for hair cystine}$$

and

$$[\alpha]_D^{20} = \frac{-8.70° \times 16.7151 \text{ gm.}}{2 \times 0.3176 \text{ gm.} \times 1.024} = -223.6° \text{ for stone cystine}$$

The concentrations are 2.67 gm. and 1.95 gm. of cystine to 100 cc. of 1 N HCl. Both concentrations, especially the latter one, fall within the limits of practically constant rotation as established by Andrews (1). Our value for the same temperature, $[\alpha]_D^{20} = -223.6 \pm 0.2°$, is identical with the maximum value of Fischer. Abderhalden (9) obtained under similar conditions $[\alpha]_D^{20} = -223.8°$ for hair cystine and $[\alpha]_D^{20} = -224.4°$ for stone cystine; figures that are again of the same order as our value. Some further support may be seen in a publication of Mörner (10) containing a very large amount of data on the isolation of cystine from a wide variety of protein substances. The highest value reported by Mörner is $[\alpha]_D = -223°$; and this value he found consistently on preparations of such different origins as horn, human hair, skin lining of the egg shell, and blood proteins. The concentrations used are all in the neighborhood of 1 per cent in 1 N HCl. As the temperature is not reported, room temperature (about 20°) may be assumed. It is difficult to conceive of materials of identical

rotations resulting from such diverse preparations unless a pure chemical individual was obtained in each case. As regards the temperature factor in the rotation of l-cystine hydrochloride in 1 N HCl, its large influence appears of special interest when compared with other substances. The earlier authors seem to have overlooked its importance in accurate and comparable determinations; and we find it first mentioned by Andrews (1) who, however, does not indicate whether a plus or a minus sign is to be used for the corrections. Evidently this has given rise to some misinterpretation, to judge from a recent publication by Gortner and Sinclair (11) in which it is stated, referring to a material of $[\alpha]_D^{20} = -201.01°$, that "Accordingly the preparation can be regarded as practically pure l-cystine." In truth, this rotation does not indicate more than 90 per cent l-cystine.

The fact that our experimental values for D and Hg rotation indicate a straight line relation between rotation and temperature may prove helpful for the future explanation of the steric changes involved.

SUMMARY.

1. By systematic fractionated reprecipitation from acid solution at different hydrogen ion concentrations, a sample of l-cystine is prepared which on further purification showed no further change in optical rotation.

2. The rotation of D and Hg light by a 1 per cent solution of l-cystine in 1 N HCl is determined over the range of 20–30°. Details about the optical method are given. For identification purposes, the Hg light is found more convenient and accurate.

3. The value found for the rotation of l-cystine, while 10° lower than the one recently reported by Andrews (1), is shown to be in agreement with the observations of several of the earlier investigators.

4. Attention is called to the unusually large temperature factor of the optical rotation and to the apparent existence of a straight line relation between temperature and rotation.

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A COMPARISON OF THE HIGHLY UNSATURATED ACIDS OF BEEF, HOG, AND SHEEP BRAINS.*

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In a preceding report (1) evidence was given for the presence in beef brains of one or more fatty acids more unsaturated and of higher molecular weight than arachidonic acid, $C_{20}H_{32}O_2$. The probable presence of an acid with five double bonds was suggested —tetracosapentenoic acid, $C_{24}H_{38}O_2$. In the present work the preparation of highly unsaturated fatty acids by a different method from fresh beef, hog, and sheep brains is described. In general the conclusions previously reported are confirmed. The highly unsaturated fatty acids of beef and sheep brains are very similar and more unsaturated than those from hog brains. The acids from hog brains give analytical results very close to the theoretical for arachidonic acid. The methyl ester polybromide, however, does not melt. A preparation of mixed methyl esters of unsaturated acids from sheep brains with an iodine number as high as 349 is described.

EXPERIMENTAL.

Preparation of Fatty Acids from Fresh Brains.

The evidence in the previous communication was based largely on the analysis of methyl esters prepared from lipids extracted from desiccated glands by benzene or from the fresh tissues by hot alcohol and ether. This procedure was tedious at its best. Consequently in the work here reported the fatty acids of the brain were prepared by direct alkaline hydrolysis of the tissue, extraction of the soaps by butyl alcohol, and acidification. 1 kilo of fresh, hashed brains was transferred to a 5 liter flask to which were added 400 cc. of 50 per cent NaOH solution. The mixture was

* Presented at the meeting of the American Chemical Society at Cincinnati, September, 1930.

cautiously heated to boiling with frequent stirring, and when thoroughly liquefied was poured into a 12 liter round bottom flask. 5 kilos of brain tissue could be worked up simultaneously and united in this flask. The boiling was continued for 1 to 2 hours. At this point 2 liters of *n*-butyl alcohol were added, and the contents of the flask were refluxed for another 30 minutes. The flame was withdrawn, and the alcohol layer allowed to separate. The sodium soaps of the fatty acids by this process go practically quantitatively into the butyl alcohol and thus can be easily separated from the large excess of alkali and other impurities. Before addition of the butyl alcohol the liquid is turbid; afterward the alkali layer is clear and of a deep reddish color. Most of the latter was removed with a siphon, and the remainder by separation in a separatory funnel. The butyl alcohol solution was boiled with an excess of HCl to decompose the soaps, the alcohol layer again separated, and the excess of butyl alcohol removed by distillation under reduced pressure. This process was hurried in order to minimize the formation of butyl esters. Water was removed along with the alcohol.

Preparation of Methyl Esters of Highly Unsaturated Acids.

The residual, dark colored fatty acids, containing cholesterol and many other substances of unknown composition, were dissolved in about 3.5 liters of dry ether, and allowed to stand for a day in the ice box. A white precipitate, nature unknown, came down and was removed by filtration. The clear, dark colored ether solution was then brominated cold, and the polybromides which came down were isolated and thoroughly washed in 250 cc. centrifuge bottles. Every precaution was taken to make this washing complete. The yield of polybromides from six lots of brains is given in Table I.

The polybromide numbers of the brains studied were essentially the same and the individual variations in those instances where more than one preparation was made were within the experimental error. It may be concluded, therefore, that there is no appreciable variation in the content of highly unsaturated fatty acids in beef, hog, and sheep brains. Nor is there any great difference in their degree of unsaturation as is shown by the constancy of the bromine content of the bromides. However if the differences in

bromine analysis are significant, they may be arranged in order of increasing unsaturation as follows: hog, beef, sheep, which agrees with the results of iodine numbers of the reduced acids, described below.

TABLE I.

Yield of Polybromides from Different Brains.

Kind of brain.	Weight of fresh tissue.	Weight of polybromide.	Polybromide No.	Bromine.
	gm.	*gm.*		*per cent*
Beef.	· 5,000	39	0.78	66.01
	4,860	48	0.98	65.83
	8,650	76	0.89	
Average.................................			0.88	65.92
Hog.	5,000	40	0.80	66.35
	5,000	44	0.88	64.45
Average.................................			0.84	65.40
Sheep.	10,750	98.4	0.92	66.31

TABLE II.

Analysis of Methyl Esters of Highly Unsaturated Fatty Acids from Beef, Hog, and Sheep Brains.

	Beef.			Hog.	Sheep.	Methyl arachidonate (theory).
Boiling point, °C.	200–258*	200–260	205–238	200–252	210–231	
	(7 mm.)	(9 mm.)	(8 mm.)	(10 mm.)	(9 mm.)	
Mean mol. wt. of acids.	325.0*	338.7	323.3	305.1	326.0	304
Iodine No.†	327.3*	343.0	339.9	324.8	349.4	318
Free acid, *per cent.*		0.13	0.6	1.5	1.7	
Polybromide No.	90.00*	102.0	92.8	73.7	103.9	
Bromine, *per cent.*	68.17*	68.25	68.02	66.68	69.48	66.78

* Reported previously.

† Hanus method, 1 hour reaction.

The polybromides were reduced by heating for 16 hours with an equal weight of zinc dust in methyl alcohol. The alcohol was removed and the methyl esters were distilled under reduced pres-

sure. The data in Table II show the analyses of the preparations of methyl esters of the highly unsaturated fatty acids, and for comparison the analysis of the preparation reported by the writer previously.

The molecular weight of the beef and sheep brain acids appears to be about 325, although one preparation for beef gave nearly 339. This is over 21 points too high for arachidonic acid. Likewise the iodine numbers of these preparations (esters) ranged from 339.9 to 349.4, 20 to 30 units too high for methyl arachidonate. The poly-bromide numbers were in all cases higher than those found for methyl arachidonate (78), while the bromine analyses of the poly-bromides were from 1.5 to 2.0 per cent too high. The similarity between the beef and sheep brain acids is obvious.

It is interesting to note, however, the difference between the hog brain esters and the others. The analytical data of these esters are decidedly similar to those of methyl arachidonate. In fact, the writers at first believed the esters from hog brains to be nearly pure methyl arachidonate until the melting point of the bromides was studied. It was found that like the esters from beef and sheep brains the methyl ester polybromide did not melt at 228–230° as would be expected with methyl octobromoarachidate, but rather shrank to a black mass with no sintering even up to 250°. There is not enough methyl arachidonate in any of these esters, therefore, to make them even partly melt at the melting point of the bromide of methyl arachidonate. These results are at present inexplicable in view of the fact that Levene and Rolf (2) have apparently iden-tified this acid in some of the phospholipids of the brain.

The one fact that should be emphasized from the data above is the decided difference between the hog brains and the others. The acids of the former are of lower molecular weight, of lower un-saturation, the polybromide number is decidedly lower, and there is less bromine in the bromides. The possibility is suggested that an isomeric arachidonic acid occurs in hog brains (and perhaps in beef and sheep brains), which upon bromination yields an iso-meric methyl octobromoarachidate which does not melt at 228–230°. The previous suggestion of the presence of tetracosapen-tenoic acid in beef brains is supported by the data given here. It may be inferred, therefore, that this acid occurs in sheep brains also. Its presence in hog brains in more than traces is question-able.

Further studies are in progress in which attempts are to be made to separate these highly unsaturated acids in order to secure additional information as to their chemical nature.

SUMMARY.

1. A method for the isolation of the fatty acids of the brain by direct alkaline hydrolysis of the fresh tissue and extraction of the soaps by butyl alcohol is described.

2. The content of highly unsaturated acids is essentially the same in beef, hog, and sheep brains.

3. The methyl esters of the highly unsaturated fatty acids of these brains have been prepared.

4. The esters from beef and sheep brains are very similar.

5. The esters from hog brains analyze very close to the thoretical for methyl arachidonate, except for the melting point of the bromide. They are less unsaturated and of lower molecular weight than the esters of beef and sheep brains.

6. The possibility of the presence of an isomeric arachidonic acid in hog brains is suggested to explain the anomaly of the melting point of the bromides.

7. Tetracosapentenoic acid probably occurs in both beef and sheep brains, but in not more than traces in hog brains.

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THE PREPARATION AND SOME PROPERTIES OF THE CRYSTALLINE METHEMOGLOBIN OF THE HORSE.

By MILTON LEVY.*

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A study of the preparation and properties of methemoglobin appeared to be important before proceeding with the further study of the methemoglobin-hemoglobin system which has been under investigation in this laboratory (1, 2). The analogies to the oxygenation reaction of hemoglobin (3) and the close relationship of hemoglobin to certain cellular oxidation-reduction systems (4) make a closer study of the system desirable particularly in regard to the shape of the potential composition curve, and the influence of pH and salt concentration on the potential. Therefore, at the suggestion of Professor Conant, the author undertook the study of crystalline methemoglobin with the object of preparing solutions of known salt content and pH, free from decomposition products, and in general suitable for the necessary measurements. Preparation of oxyhemoglobin solutions of the desired type is illustrated by the solubility determinations of Cohn and Prentiss (5).

Preparation of Crystalline Methemoglobin.

Crystalline methemoglobin has been prepared by two methods. These are modifications of procedures described in the literature One depends on the oxidation of the hemoglobin by potassium ferricyanide and has been previously used by Huffner and Otto (6). and Jaderhölm (7). The other depends on the autooxidation of oxyhemoglobin in the presence of alcohol and has been used by Huffner and Otto (6) and by Zeynek (8). Haurowitz (9) has prepared crystalline horse methemoglobin by both methods.

The starting material in either case was crystalline oxyhemoglobin paste prepared by the method of Ferry and Green (10), except

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that laking was accomplished by freezing.   The crystals were washed with distilled water or any desired buffer solution.   Two modifications of the ferricyanide procedure will be described in detail and one of the alcohol method.

The paste of oxyhemoglobin crystals is diluted with about one-fourth its volume of water or buffer solution and stirred until a readily flowing mass free of lumps is obtained.   About 300 cc. of this suspension are placed in a tared liter tonometer and deoxygenated by repeated evacuation, shaking, and refilling with purified nitrogen to cut down foam.   The crystals dissolve and a solution of hemoglobin free of oxygen is obtained.   The oxygen capacity of the solution is determined by dilution of a sample with sufficient water to hold the oxyhemoglobin in solution and analysis by the Van Slyke-Neill manometric method (11) after aeration.   From the oxygen capacity the concentration of hemoglobin may be calculated; it is usually 14 to 16 milli-equivalents per liter.   The tonometer and its contents are weighed and the weight of the solution calculated.   An amount of potassium ferricyanide equivalent to the hemoglobin in solution, calculated on the basis of a specific gravity of 1 is dissolved in 10 cc. of water and introduced into the tonometer.   The method of calculation provides an approximate excess of ferricyanide of 10 per cent.   The solutions are mixed by shaking and rolling the tonometer.   Crystallization appears to occur immediately but the mixture is always placed in the cold room overnight.   The crystals are removed and washed by centrifuging.

The deoxygenation process is the most time-consuming step and, in order to avoid this, another procedure which has certain advantages has been used.   In this process the paste of oxyhemoglobin is brought into solution by the slow addition of N KOH with vigorous mechanical stirring.   The alkaline oxyhemoglobin solution so obtained is centrifuged to remove a small amount of insoluble matter.   The solution is analyzed for its oxygen content and a solution containing a 10 per cent excess of potassium ferricyanide and sufficient KOH and KH_2PO_4 to give, with the KOH already added, the desired pH and ionic strength in the final mixture, is added slowly and with efficient mechanical stirring.   Methemoglobin crystallizes from the solution on standing overnight and is centrifuged and washed.

In the presence of alcohol oxyhemoglobin undergoes spontaneous transformation into methemoglobin. This transformation is accelerated by small amounts of acid (8) and at temperatures of 35–37° (9). However, both these conditions also increase the rate of cathemoglobin formation necessitating a proper choice of conditions in order to obtain methemoglobin in predominating quantity and in a reasonable length of time.

The paste of oxyhemoglobin crystals is diluted with sufficient water to give a fluent mass free of lumps and 10 per cent of its volume of 95 per cent alcohol is added with vigorous stirring. The mixture is then incubated for 70 to 80 hours at 36–38°. So far we have not been able to obtain preparations entirely free of cathemoglobin or oxyhemoglobin by this method although preparations suitable for the determination of solubility may be obtained. These are only freed of oxyhemoglobin after a number of washings and in some instances preparations were obtained which on washing seemed to give solutions containing constant quantities of both oxyhemoglobin and methemoglobin, although both were present in concentrations below their ordinary solubilities. This phenomenon will be investigated further. The methemoglobin obtained by means of alcohol may be dissolved by the addition of alkali and centrifuged to remove cathemoglobin, although since this is insoluble around neutrality, its presence does not disturb the solubility measurements except in so far as it increases the volume of retained solutions. The alkaline solution may be brought to crystallization by neutralizing the base added. The crystals so obtained contain much less oxyhemoglobin than the original.

Properties of the Crystals.

Crystals prepared by all of the above methods are microscopic, coffee-brown, rhombic platelets occasionally appearing hexagonal because of incomplete growth at the acute angles. They are very similar in shape and habit to the oxyhemoglobin crystals of the horse, which perhaps is not surprising in view of the large common protein fraction. The methods used in preparing the crystals through oxidation with ferricyanide exclude the possibility of pseudomorphism, although the method with alcohol does not. The crystals are completely soluble in alkali and may be repre-

cipitated by neutralization. The recrystallization may be accomplished by slowly adding a strong acid in amounts equivalent to the base or until rhombic platelets begin to appear on slight evaporation of a drop of the solution on a microscope slide. As with oxyhemoglobin solutions, needle-like crystals appear at a certain stage of incomplete neutralization. Apparently these are the needle-like crystals which Haurowitz (9) described.

Exhaustive washing of the crystals near neutrality leaves only a small amount of a white amorphous residue which is not protein. In the case of those samples made by the alcohol procedure and not recrystallized varying amounts of cathemoglobin remain undissolved. The solutions prepared around neutrality do not contain measurable amounts of cathemoglobin or other decomposition products, for the O_2 and CO capacities after reduction are equal within the limits of error. If an appreciable quantity of cathemoglobin had been present in the solution, the CO capacity would have been greater than the O_2 capacity (12).

Solubility of the Crystals.

One criterion of the purity of the solid phase is its constant solubility; and no other well recognized means is available in the case of proteins. Therefore, the solubility of methemoglobin in a number of phosphate buffers was determined, and at Dr. Cohn's suggestion some of the particular buffers used by Cohn and Prentiss (5) were employed in order to make a comparison. These compositions are based on data given by Cohn (13). The pH values of the buffers were checked by means of the hydrogen electrode.

In determining the solubilities the crystals are first washed a number of times with the desired buffer, only sufficient contact being allowed to dilute the mother liquors. Between each addition of buffer the mixtures are centrifuged and the liquid poured off. In this way as much as possible of the liquid retained with the crystals is replaced by the desired buffer. Nevertheless, it is still necessary to wash five or ten times, stirring during a period of 24 hours before solubility becomes constant. After the preliminary washing the crystals are placed in a 250 cc. centrifuge bottle fitted with a mechanical stirrer, and enough buffer solution added to cover the crystals (20 to 25 cc.). The bottles are then

immersed in an ice water bath kept in a cold room at 3–4°.   After stirring for 24 hours the bottle is removed and centrifuged in the cold room for approximately 10 minutes.   The crystals and solution separate sharply, but the solution is always poured through a dry filter paper, the first 20 or 30 drops being rejected as they come through.   A fresh portion of solvent is added and the mixture stirred for another 24 hours or longer.

The samples are analyzed for methemoglobin by reduction with titanous chloride and subsequent aeration according to the method

TABLE I.

Solubility of Methemoglobin at 0°.

Solubilities are measured in gm. per 100 cc.

pH	6.6	6.6	6.6	6.8	7.0
Ionic strength, μ..............	0.25	0.64	1.0	0.25	0.25
Total phosphate, M..........	0.136	0.326	0.495	0.121	0.110
Mol fraction of K_2HPO_4....	0.419	0.482	0.509	0.537	0.627
Ferricyanide method.	6.45	8.93	10.48	5.31	· 5.54
	6.27	8.82	10.57	5.49	5.44
	6.33	8.88	10.41	5.27	6.05
	6.17			5.50	
				5.24	
Average............	6.32	8.88	10.48	5.36	5.67
Alcohol method.	6.32				5.91
	6.33				5.66
	6.41				
	6.22				
Average............	6.32				5.78

of Conant, Scott, and Douglass (12).   This method was adopted because it is as convenient as the Van Slyke method (14) and at the same time is more specific for methemoglobin (12).   It also has the advantage over a nitrogen determination in being undisturbed by the presence of other proteins.

The criterion of equilibrium is a constancy in the methemoglobin contents of successive samples.   In general only the last two values are low after equilibrium is attained.   The solid phase at this time is probably too small to completely saturate the solution in the time allowed.

The results of the solubility determinations are shown in Table I. The solubilities are expressed in gm. of methemoglobin per 100 cc. of solution. These are calculated from the oxygen capacity after reduction on the basis that 1 cc. of oxygen combines with 0.746 gm. of hemoglobin (15). In Table I the determinations listed under "Ferricyanide method" were performed on crystals made by that method; under "Alcohol method" the crystals were made by the method with alcohol as described above.

The pH of minimum solubility is seen to be 6.8 at a constant ionic strength of 0.25. It may be noted that the minimum of solubility for oxyhemoglobin was found by Cohn and Prentiss (5)

TABLE II.

Solubility of Methemoglobin.

pH	6.6	6.6	6.6
μ	0.25	0.64	1.0
$\sqrt{\mu}$	0.5	0.8	1.0
S, gm. per l.	63.2	88.8	104.8
Log S	1.800	1.948	2.020
Log $\frac{S}{S_0}$ (calculated)	0.571	0.727	0.800
Log S_0	1.229	1.221	1.220

to be at pH 6.6 and to differ from the isoelectric point which is given as pH 6.78 ± 0.03 (16).

Cohn and Prentiss (5) found that the equations of Debye (17) are adequate to describe the effect of salt concentration on the solubility of oxyhemoglobin over the range of ionic strength 0 to 1.0, if this substance is considered to be a bivalent ion, a bi-bivalent compound, or a uni-quadrivalent compound. The equations are satisfied even though they are based on the assumption, which is certainly untrue for oxyhemoglobin, that the ions have a mean effective diameter of the order of 10^{-8} cm. Cohn and Prentiss (5) derive as the equation governing the change in solubility of oxyhemoglobin with salt concentration

$$\log \frac{S}{S_0} = \frac{2\sqrt{\mu}}{1 + 1.5\sqrt{\mu}}$$

in which S is the solubility in a phosphate buffer of ionic strength μ, and S_0 is the solubility in a buffer of the same pH but zero ionic

strength. The latter is of course a hypothetical solution. The factor 2 is a combination of the assumed valence product (product of the valencies of the positive and negative ions) 4, and the factor 0.5 which appears as a constant in the Débye equations. The factor 1.5 in the denominator is a constant depending on the mean effective diameter of all the ions in the solution and a reciprocal distance related to the decrement of density of charge in the solution as the distance from the source of charge increases.

The solubility of methemoglobin as it varies with the ionic strength of the phosphate buffer may be described by the same equation. This is shown by the constancy of log S_0 in Table II. Methemoglobin is, therefore, of the same *apparent* valence type as oxyhemoglobin.

Stability of Methemoglobin.

Our methemoglobin solutions remain stable in the cold room (2–4°) for several weeks. That is, neither reduced nor oxyhemoglobin is formed and methemoglobin does not disappear, although no precautions beyond ordinary cleanliness were taken to prevent bacterial contamination. In determining solubilities the crystals of methemoglobin are exposed to air for as long as 20 days with continuous mechanical stirring, and the oxygen contents of the solutions do not exceed the expected oxygen solubility. It is proper to conclude from this that in the presence of oxygen, methemoglobin is not reduced in significant quantities. These facts, which are further supported by experiments at room temperature to be presented below, are mentioned because Heubner and Rhode (18) postulate a direct and observable equilibrium between methemoglobin, oxygen, and oxyhemoglobin, writing the equation

$$4HbO_2 + 2H_2O = 4HbOH + 3O_2 \tag{1}$$
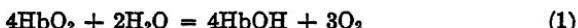
in which HbO_2 represents oxyhemoglobin and $HbOH$, methemoglobin. It will be shown below that the equilibrium in this reaction favors the presence of methemoglobin and O_2 to such an extent that it is impossible to observe formation of oxyhemoglobin. The same calculations also demonstrate that oxyhemoglobin is thermodynamically an unstable compound with respect to the formation of methemoglobin.

For these calculations it is convenient to use more modern

formulæ for oxyhemoglobin and methemoglobin, with an equation which probably more closely represents the true reaction.

$$4H_3(HbO_2) \quad = \quad 4H_2Hb + 3O_2 + 2H_2O \qquad (2)$$

Oxyhemoglobin   Methemoglobin
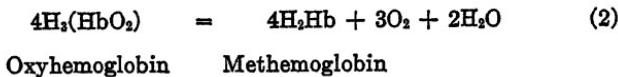
Using this equation, we are readily able to calculate the approximate free energy of the reaction, and from this estimate the position of equilibrium.

Conant and Fieser (2) measured the normal potential of the hemoglobin-methemoglobin system and found it to be approximately +0.120 volts at a pH of 7.4, selecting the normal hydrogen electrode as the standard.   Therefore, for the reaction

$$4H_2Hb \quad + 2H_2 = \quad 4H_3Hb \qquad \Delta F = - NEF \qquad (3)$$

Methemoglobin   Hemoglobin $= - 4 \times 0.120 \times 23074$

$= - 11,275$ calories
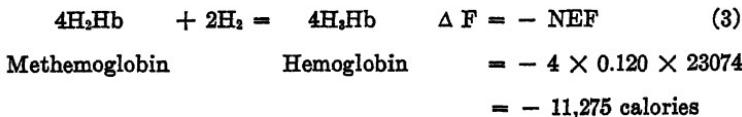
The free energy of the oxygenation reaction

$$H_3Hb \quad + O_2 = \quad H_3HbO_2 \qquad (4)$$

Hemoglobin   Oxyhemoglobin
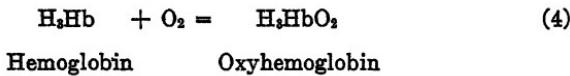
may be approximately calculated from the equilibrium data of Ferry and Green (10).   Thus in one experiment at pH 7.38 they find that at an O_2 pressure of 11.2 mm. and at 25°, hemoglobin is 70 per cent saturated with oxygen.   From these data the free energy change of the reaction is given by the equation (19).

$$\Delta F = - RT \ln \frac{70}{30} \times \frac{760}{11.2}$$

$$= - 1370 \log 158.0$$

$$= - 1370 \times 2.2 - - 3020 \text{ calories}$$

Data at other O_2 tensions give differing values for the free energy, but the results fall between -2000 and -4000 calories. We may take -3000 calories as the average.   We may then add Equation 3 obtained from potential measurements and Equation 4 obtained from the oxygenation equilibrium, multiply by suitable coefficients, and obtain

$$4H_2Hb \quad + 2H_2 + 4O_2 = \quad 4H_3HbO_2 \quad \Delta F = -23,300 \text{ calories} \quad (5)$$

Methemoglobin   Oxyhemoglobin
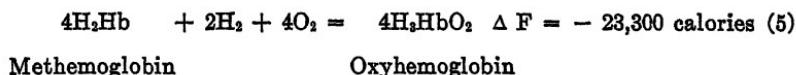
Lewis and Randall ((19) p. 485) give for the reaction

$$H_2 + 0.5O_2 = H_2O \qquad \Delta F = -56,560 \text{ calories} \qquad (6)$$
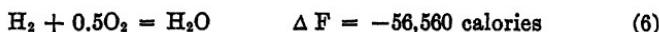
By subtracting Equation 6 from Equation 5 with suitable coefficients, Equation 7 results.

$$4H_2Hb + 3O_2 + 2H_2O = 4H_3HbO_2 \qquad \Delta F = +89,820 \text{ calories} \quad (7)$$

We may calculate from this the concentration of oxyhemoglobin which would be in equilibrium with 10 per cent solution of methemoglobin and 1 atmosphere pressure of oxygen

$$89,820 = -1370 \log \frac{(H_3HbO_2)^4}{(H_2Hb)^4 \, pO_2^3 \, (H_2O)^2}$$

pO_2 and (H_2O) are both one and may therefore be dropped and the equation becomes

$$89,820 = -4 \times 1370 \log \frac{(H_3HbO_2)}{(H_2Hb)}$$

$$\frac{89,820}{-5480} = -16 = \log \frac{(H_3HbO_2)}{(H_2Hb)}$$

or

$$(H_3HbO_2) = 10^{-16} (H_2Hb)$$

Approximately, therefore, the concentration of oxyhemoglobin which would be in equilibrium with a 10 per cent solution of methemoglobin under a pressure of 1 atmosphere of O_2 is 10^{-15} gm. per 100 cc., certainly undetectable by any known method. To put it another way, the oxygen pressure necessary to convert half the methemoglobin to oxyhemoglobin is about 100,000 atmospheres. Even if O_2 and methemoglobin were in equilibrium, as postulated by Heubner and Rhode (18), the detection of such an equilibrium is impossible. The results are quite different if a stronger reducing agent than water be present and we believe that the oxyhemoglobin which was observed by Heubner and Rhode was due to unknown reducing agents in their solutions.

A number of experiments were performed in order to show the stability of methemoglobin and to verify the results of Heubner and Rhode. A solution containing 5.3 per cent of methemoglobin in a phosphate buffer was diluted with 0.1 volume of the various solutions listed in Table III. The O_2 content after 48 hours in the cold room and after a subsequent 48 hours at room temperature, showed that something in serum and in washed laked cells was able to reduce methemoglobin at room temperature. Since Heubner and Rhode made hemoglobin solutions by directly laking cells, Experiment 3 corresponds to their conditions and results.

TABLE III.

Stability of Methemoglobin.

Experiment No.	Substance added.	O_2 content.		
		Beginning.	48 hrs. in cold room.	48 hrs. at room temperature.
		vol. per cent	*vol. per cent*	*vol. per cent*
1	Water.	0.54	0.40	0.72
2	Serum.	0.55	0.76	2.11
3	Laked cells.*	1.52	1.60	1.90
4	Crystalline Hb solution.	1.37	1.50	0.60
5	$K_4Fe(CN)_6$.†	0.73	0.77	0.52
6	Glucose.†	0.75	0.79	0.53
7	Lactic acid.†	0.74	0.80	0.50
8	Uric acid.†	0.72	0.81	0.73

* 1 volume of cells plus 4 volumes of water.

† Added in amounts equivalent to the methemoglobin.

Any one of a number of reducing agents in the serum or cells may be responsible, but since crystallizing the oxyhemoglobin removed the responsible agent, our interest did not extend further.

SUMMARY.

1. Three methods of preparing crystalline methemoglobin of the horse are described.

2. The solubility of horse methemoglobin in a number of phosphate buffers has been determined.

3. The solubility depends on the ionic strength of the buffer in the same manner as does the solubility of oxyhemoglobin.

4. Oxyhemoglobin is shown to be thermodynamically unstable with respect to the formation of methemoglobin and oxygen, and any detectable amount of oxyhemoglobin spontaneously appearing in methemoglobin solutions owes its origin to unknown reducing agents and not to the appearance of an equilibrium with O_2.

I wish to record my thanks to Professor J. B. Conant for suggesting this problem and for his continued interest, and to Professor E. J. Cohn and his coworkers for valuable advice in connection with the solubility determinations.

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SOFT PORK STUDIES.

IV. THE INFLUENCE OF A RATION LOW IN FAT UPON THE COMPOSITION OF THE BODY FAT OF HOGS.

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It is generally recognized that the fattening animal stores a more saturated type of body fat when the feed is low in ether-soluble substances than when oils such as corn, peanut, and soy bean are present even in moderate quantities. Recent work with hogs (1) has shown that the wide variations in composition of the body fat are in general closely related to the quantity and degree of unsaturation of the oil ingested in the feed. The most saturated fat was found in hogs fattened on the ration lowest in ether extract. Anderson and Mendel (2) and Eckstein (3) found that the addition of fatty substances to an otherwise fat-free ration altered the composition of the body fat of rats. In general the body fats resembled the ingested oils or other fatty substances as judged by the iodine numbers. Fat-free rations, high in carbohydrates as well as in proteins, produced body fats in rats with iodine numbers of 60 to 70. These values correspond to those found in the fat of hogs fed on rations moderately low in ether extract such as corn with tankage (4).

The firm body fat which occurs in hogs, cattle, sheep, and other animals when feed fat is not an important constituent in the ration is made up largely of oleic, stearic, and palmitic acids combined in various glyceride combinations. Other fatty acids occur in relatively small amounts in the usual case. Contrary to statements frequently noted in articles and books on fats, lards may show a wide range in saturation, usually the result of the ingestion

and utilization of feed oils.   The iodine numbers may vary from
approximately 30 to 100 and over.   These extremes are the result
of widely different rations such as copra cake (5) on the one hand
and peanuts or soy beans on the other.   In the case of the oily
feeds such as peanuts or soy beans, linolic acid is known (1) to
rank in importance with the three acids previously named.   It is
noteworthy that a vegetable fat such as that in copra meal should
produce a more saturated fat than a ration low in fat where the
iodine numbers usually range from 50 to 60.   Such values have
been reported in German and Danish investigations in which the
rations fed were composed of potatoes, barley, skim milk, and
other feeds low in fat as well as in the present investigations
when brewers' rice was used as the basal feed.

Shortly after the soft pork investigations were undertaken 10
years ago, by the United States Department of Agriculture and
cooperating state experiment stations, the production of extremely
firm carcasses on a rice by-product feed known as brewers' rice was
noted.   This feed has consistently produced firmer carcasses (1)
than have other materials, including corn, which contained more
oil (ether extract).   The fact that other feeds low in oil such as
sweet potatoes, hominy, and barley (unpublished results) have
produced similar although less striking results supports the view
that the lack of oil in the feed is the principal influence toward
production of firm body fat on these foodstuffs.

Following the experiments previously reported (4) on the pro-
gressive hardening of hogs on rations of corn with non-softening
supplements in which the probable influence on firmness of the
oil contained in the corn was pointed out, the present experiment
with brewers' rice as the basal feed was undertaken.   Attention
has been directed in this experiment to a study of the quantity
and composition of the body fat of hogs at successive stages of
growth.   The hogs were reared on a ration containing approxi-
mately 0.5 per cent of ether extract.   Not only were the experi-
mental animals grown from weaning on this ration but their dams
received a similar ration during the periods of pregnancy and
suckling.   Body fat was produced at a normal rate varying in com-
position only to a small extent from weaning to market weight.
As compared to the results on corn rations, which contained
approximately 4 per cent of ether extract, the progressive harden-

ing was much less pronounced and the fat more saturated in the present experiments. The predominating fatty acids were oleic, palmitic, and stearic. Linolic acid was present in small amounts, but the quantity apparently bore no relation to the increase in total fat. During the late stages of growth, the conversion of non-fatty constituents, mainly carbohydrates, into fat was remarkably high although it should not be inferred that it was more rapid than normally occurs on a properly balanced ration.

Plan and Procedure of the Experiment.

Two pregnant sows, one a Chester white and the other a Duroc-Jersey, were placed on a ration of brewers' rice, alfalfa meal, and blood meal balanced to an appropriate nutritive ratio. The sows were purposely selected because of their thin condition in order to insure a preponderance of firm fat in the tissues during the suckling period. Evidence that this was accomplished was obtained later when one of the sows was slaughtered after the pigs had been weaned. The carcass was found to have a firmness grade of "hard," and an iodine number analysis on the back fat gave a value of 52.9. The two sows made rapid gains in weight and in fatness. They farrowed large, normal litters. From the seventeen pigs alive at the age of 6 weeks, thirteen were used in the experiment. The pigs were allowed access to the feed mixture given the dams during the suckling period and no attempt was made to obtain feed consumption records until after weaning.

Two pigs were killed at the midway point of the suckling period and two more at weaning time. Beginning shortly after weaning the remaining nine pigs were hand fed in individual feeding compartments twice daily. The ration, consisting of brewers' rice, blood meal, alfalfa meal, and mineral mixture, was weighed out at each feeding. It was the intention to change the proportion of rice and blood meal at intervals to adjust the nutritive ratio according to requirements of the hogs. The alfalfa meal was kept at 8.5 per cent and the mineral mixture at 1.5 per cent of the total mixture. From weaning until 110 pound weight was reached, the nutritive ratio was approximately 1:4.7. When the mixture was changed at the 110 pound weight the amount of blood meal was inadvertently increased so that the nutritive ratio was approximately 1:4. This was corrected within a few weeks and the nutritive ratio fed until the end of the experiment was 1:5.7.

After the slaughter of the four pigs as mentioned, the remainder were slaughtered in pairs at weights of approximately 75, 110, 170, and 240 pounds.   The remaining one was slaughtered at a weight of 300 pounds.

The same general plan of slaughter and analysis as used in earlier work of similar nature (4) was followed.   The entire body was analyzed for protein, water, fat, and ash in five fractions, namely, blood, cleaned organs and alimentary tract, skin, bone, and meat. After calculating the constituents in each fraction they were totaled and the loss in shrinkage from "hot" to "cold" carcass weight added to the moisture total.   Calculation of constituents was thus made on the total empty weight minus hair and scurf.   The weight of hair was obtained in a number of cases.   The analysis of one composite sample showed 87 per cent protein.   The protein in the hair was found to be approximately 4.5 per cent of the total protein in the body.

The carcasses of all hogs killed subsequent to weaning were chilled for 2 to 3 days and then graded for firmness according to the grading system used in the cooperative soft pork investigations.   Samples of back and leaf fats were rendered, filtered, and analyzed for fat constants.   Special composite fat samples representative of the entire body were prepared by heating 5 pound lots of ground meat on the steam bath for 2 to 3 hours, then, after expressing the fat and water liquid from the meat residue by draining and pressure, the melted fat was separated in a separatory funnel and filtered.   The detailed work on fatty acid separations was made on these composite fat samples.

Experimental Results.   Growth and Feed Consumption.

A record of the feed consumption was kept on the nine hogs continued on experiment subsequent to weaning.   These animals were slaughtered at the weight intervals chosen for study as indicated in Table I.   The increase in rate of gain with increase in weight is in keeping with the usual progressive increase in rate of gain for hogs of this weight range.   It will be noted that the feed consumption for unit gain is given by weight intervals as well as for the entire period of the experiment.   There was an increase in the amount of feed consumed per pound of gain during the interval of growth from 110 to 170 pounds, when the protein content of the

TABLE I.

Growth and Feed Consumption of Individual Hogs.

Hog No. and breed.*	Period I, group feeding.				Period II, on individual feeding test.							
								Feed consumed per lb. of gain by weight intervals.				
	Birth weight.	Weight at close of period.	Days on test period.	Average daily gain.	Weight at close of feeding.	Days on test period.	Average daily gain.	Beginning of period to 75 lbs.	75-110 lbs.	110-170 lbs.	170 to close of test.	Entire period.
	lbs.	*lbs.*		*lbs.*	*lbs.*		*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*
1 C.	2.75	18.5†	35	0.45								
2 D.	2.50	22.7†	46	0.44								
3 C.	2.75	31	61	0.46								
4 D.	3.00	44	72	0.57								
5 "	3.25	61	85	0.68	75	25	0.56	3.28				3.28
6 C.	3.25	42	74	0.52	75	37	0.89	2.64				2.64
7 "	3.00	47	74	0.60	110	60	1.05	2.36	2.90			2.66
8 D.	3.25	52	85	0.57	110	60	0.97	2.87	2.90			2.89
9 "	3.25	58	85	0.64	170	114	0.98	3.53	2.62	4.47		3.75
10 C.	3.00	35	74	0.43	166	131	1.00	2.96	3.41	4.94		3.97
11 "	2.75	39	74	0.49	253	171	1.25	3.11	2.77	5.08	3.48	3.72
12 "	3.00	37	74	0.46	246	171	1.22	2.95	2.77	5.04	3.82	3.83
13 D.	2.25	56	85	0.63	299	171	1.42	2.89	2.40	4.71	3.00	3.31
Average....................................								2.96	2.82	4.85	3.34	3.34

* The letters attached to the numbers refer to the breed as follows: C., Chester white; D., Duroc-Jersey.

† Slaughtered before the close of Period I.

TABLE II.

Composition of the Feeds.

Feed.	Moisture.	Protein.	Ash.	Ether extract.	Crude fiber.	N-free extract.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Brewers' rice.....................	13.7	7.2	0.7	0.5	0.5	77.4
Blood meal......................	15.0	79.0	6.4	0.3		
Alfalfa meal.....................	9.2	9.3	10.1	1.2	30.2	39.9

Soft Pork Studies.   IV

ration was high.   However, the daily feed intake did not show an abnormal increase.

The composition of the feeds is given in Table II.   As already stated these feeds were mixed to give a certain nutritive ratio at a given period in the growth of the animals.   On a percentage basis the protein content ranged from 12 to 17, the carbohydrates from 66 to 68, while the ether extract was approximately 0.5 throughout the experiment.   These figures at once indicate that only a small proportion of the body fat could have been derived from the

TABLE III.

Composition of the Entire Body.

Hog No.	Age at slaughter.	Weight at slaughter.	Total analyzed weight.	Composition.				Total weight of fat.
				Water.	Protein.	Fat.	Ash.	
	days	*lbs.*	*lbs.*	*per cent*	*per cent*	*per cent*	*per cent*	*lbs.*
1	35	18.5	17.7	67.61	15.35	13.60	3.44	2.41
2	46	22.7	20.8	64.95	14.71	17.02	3.52	3.53
3	61	29	26.7	66.82	15.78	14.16	3.24	3.78
4	72	41	38.0	62.15	14.42	20.62	2.81	7.83
5	110	73	65.7	57.29	14.77	25.05	2.89	16.44
6 .	111	73	66.5	60.30	15.54	21.63	2.53	14.34
7	134	110	100.1	57.25	14.68	25.57	2.50	25.60
8	145	110	100.1	54.42	14.05	28.86	2.67	28.90
9	199	170	159.3	46.50	12.78	38.24	2.50	60.87
10	205	166	154.8	45.20	12.97	39.52	2.29	61.14
11	246	243	236.4	45.76	12.32	39.35	2.57	92.91
12	246	244	231.9	46.41	13.49	37.66	2.44	87.43
13	257	282	275.8	40.82	11.76	44.92	2.50	123.90

ingested fat and that much of it necessarily must have been synthesized from the carbohydrates.

The analyses given in Table III on the composition of the entire body give the per cent of water, protein, fat, and ash in the entire body, hair excepted.   The total protein including the hair may be calculated, since it was found, as previously mentioned, that the protein in the hair constituted approximately 4.5 per cent of all other protein in the body.   The fat content of these hogs was somewhat above the average for the corn-fed hogs in the earlier experiment up through the 170 pound group.   Indeed at this weight the rice-fed hogs were much above the average.   At

heavier weights the animals herein reported were somewhat below the average. However, it was not enough to be significant. Indeed, observations on carcasses of hogs similarly fed have frequently indicated excessive fattening. The results on Hog 13 are of particular interest in illustrating the rapid synthesis of fat which can take place. This animal made the most rapid gain and most efficient feed utilization (see Table I) for the entire experimental period. When slaughtered at the age of 257 days it had

TABLE IV.

Grades and Fat Constants of Individual Hogs.

Hog No.	Carcass grade.	Refractive index, — 40°.	Iodine No.	Melting point.	Titer test.	Saponification No.	Reichert-Meissl No.	Polenske No.
				degrees				
1		1.4591	63.6	25.6	37.2	201.9	1.00	
2		1.4593	65.7	26.0	36.4	200.0	1.00	2.00
3		1.4591	66.1	24.6	35.6	200.0		2.05
4		1.4591	64.9	25.8	35.7	201.3		1.25
5	Medium soft.	1.4590	61.2	34.8	37.5	201.2	0.35	0.80
6	" "	1.4588	56.5	37.4	38.7	199.4		0.80
7	" hard.	1.4584	57.4	35.8	37.9	199.8		0.85
8	Hard.	1.4582	54.3	39.7	39.3	200.1		1.00
9	"	1.4589	57.6	32.8	37.4	195.5	0.30	0.50
10	"	1.4583	55.6	35.4	37.5	195.1	0.18	0.42
11	"	1.4584	53.3	32.8	38.0	195.8	0.51	0.54
12	"	1.4587	57.4	33.0	37.1	194.6	0.36	0.42
13	"	1.4584	55.1	37.6	38.1	195.0	0.18	0.81
Chester white dam.		1.4580	52.9					

stored 123.9 pounds of fat or at the average rate of 0.49 pound (220 gm.) per day. Wierzuchowski and Ling (6) in a calorimetric study of fat production found a daily average production of 98 gm. of fat in a pig weighing 13.5 kilos (30 pounds). Their animal was on test for 47 days (from the 70th to 117th day of its life) and during this time grew from a weight of 8.5 to 18.1 kilos (19 to 40 pounds).

Litter mates of Hog 13 which were killed at weights of 22.7 and 41 pounds (10.3 and 18.6 kilos) showed a difference in fat content of 4.30 pounds. With this as a basis for calculation, Hog 13 which

increased in weight from 20 to 40 pounds between the 36th and 56th days of its life stored fat at the average daily rate of 0.22 pound (101 gm.), which is 3 gm. higher than that reported by Wierzuchowski and Ling.   During the last 15 days of the experimental feeding period this animal gained 46 pounds, and, according to estimate, stored approximately 1.85 pounds (840 gm.) of fat daily.

Composition of Body Fat.

The fat constants determined on the individual samples of lard rendered from the composite meat samples are given in Table IV. The gradings for firmness of the chilled carcass are also given for the nine hogs killed subsequent to weaning.   Hogs 5 and 6 were graded medium soft.   Experience in the grading of animals of light weight such as these has shown that the grading does not follow the composition of the fat so closely as it does in heavier animals where a greater proportion of the carcass is adipose tissue. Hog 7 graded medium hard and the remainder all graded hard. The refractive index, iodine number, and titer test indicate a hard fat even in the young pigs.   The melting point values show little if any correlation with the other analyses.

There was a tendency for the fat to increase in firmness with increasing weight of the animal.   However the maximum firmness was apparently reached in Hogs 7 and 8.   As compared with earlier results on hogs fed a basal ration of corn, the fat constants as given in Table IV show that the fat of the rice-fed hogs was not only more saturated at all weights but particularly so at the lighter weights.   There was also a shorter and less abrupt course in the progressive hardening of the hogs in the present experiment than in the former.   It is believed that this can safely be attributed to the low amount of ether extract in the feed.   At the same time it lends additional support to the idea previously expressed in connection with the progressive hardening of hogs fed on a corn ration relative to the indirect rôle of the ingested fat in determining this increase in firmness with increase in the rate of fattening.   The saponification, Reichert-Meissl, and Polenske values are indicative of a greater proportion of lower molecular weight acids in the young animals than in the older ones.

Perhaps the most important question raised in connection with

the experiment was that of the distribution of the various fatty acids occurring in the adipose tissues.   Lead salt-ether separations of the saturated and unsaturated acids were made on the fat samples from the individual hogs.   The unsaturated fractions were brominated and small quantities of an octabromide recovered. This amounted to 0.11 per cent in the fat of Hog 2, while the other hogs, particularly those toward the end of the experiment, contained considerably less.   On the basis of previous analyses (1) this octabromide was assumed to be arachidonic acid.   Eckstein

TABLE V.

Fatty Acid Distribution in Body Fat.

Hog No.	Unsaturated acids.				Saturated acids.			
	Total.	Oleic.	Linolic.	Arach-idonic.	Total.	Myristic.	Palmitic.	Stearic.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	64.0	57.9	6.9	0.05	31.2			
2	63.0	53.9	9.0	0.11	32.1			
3	65.0	57.4	7.5	0.07	29.8			
4	63.5	55.5	7.9	0.07	31.6			
5	61.2	54.9	6.2	0.07	33.3	1.1	24.2	8.0
6	58.7	55.1	3.6	0.03	36.4			
7	60.2	53.9	4.3	0.05	35.3	0.7	26.1	8.5
8	57.6	55.2	2.4	0.06	37.4			
9	60.1	58.0	2.1	0.04	35.1			
10	60.7	59.7	1.0	0.02	34.6			
11	58.6	57.8	0.8	0.03	37.0	1.0	25.0	11.0
12	62.0	59.4	2.6	0.04	33.6			
13	60.2	58.9	1.3	0.02	35.3	0.7	24.3	10.3

(7) as well as Wesson (8) have reported its presence in body fat. With the exception of this small amount of arachidonic acid the unsaturated fraction consisted of oleic and linolic acids.   The proportions were calculated from the iodine numbers and are given in Table IV as per cent of the total fat.

It will be noted in Table V that the oleic acid varied from 53.9 to 59.7 per cent.   However, there was no consistent change except a tendency to increase with increasing weight of animal.   The linolic acid shows a pronounced decline from 9.0 per cent for Hog 2 to 0.8 per cent for Hog 11.   The low values found in Hogs 8 to 13

correspond to those previously reported (1) for hogs fed a similar ration of brewers' rice and supplements.   The minimum content reported was 1.2 per cent, which occurred in a composite sample of back fat taken from hogs fed brewers' rice, tankage, and skim milk. It was suggested that the quantity occurring in the body fat was less than that ingested in the feed consumed by the animals and could have been derived from this source instead of direct synthesis. Results from various feeding experiments support the view that linolic acid is not synthesized and deposited to any great extent in the fattening hog but can generally be traced to the ingested fat.

The fact that a relatively greater proportion occurred in the young animals in the present experiment seems to indicate that synthesis of linolic acid or conversion of other ingested fatty acids to this acid followed by deposition does take place to a certain extent at least in young animals.   The calculated amounts in Hogs 4, 5, and 6 (from Tables III and IV) range from 0.5 to 1.0 pound.   This quantity was not greatly exceeded in the remaining animals except Hogs 12 and 13, which show approximately 2.25 and 1.60 pounds, respectively.   These figures indicate that little if any synthetic linolic acid was deposited after the hog reached a weight of 75 pounds.   The calculated amount of ether extract in the feed consumed from weaning to slaughter was approximately 4.5 pounds for Hogs 11, 12, and 13.   Analyses on this ether extract gave an iodine number of 80.3 and a saponifiable matter content of 72.6 per cent.   Since this was derived for the most part from the brewers' rice, the composition of this fatty acid material was assumed to be similar to that in rice oil as given by Jamieson (9). Thus estimated, the oleic and linolic acid content was 32 and 29 per cent, respectively.   Calculation of the quantities of linolic and oleic acids ingested by Hogs 11, 12, and 13 gives 1.4 and 1.3 pounds, respectively.   This amount of linolic acid was sufficient to account for that deposited in the body subsequent to weaning.

In considering the possible explanation of the relatively high proportion of linolic acid in the small animals, one possible source was the sow's milk.   Milk fat normally contains little or no unsaturated acids other than oleic.   A small sample of milk was obtained from one of the dams at the close of the suckling period when she was slaughtered.   The iodine number of the fat was 51.6, which is much higher than normally found in sow's milk.   How-

ever, an attempt at determination of unsaturated acids did not reveal any linolic acid nor the probability of any appreciable quantity being present. The sample was too limited to admit of extended analysis. It has been frequently noted that the outermost adipose tissue lying directly under the skin as well as the fat in the skin is more unsaturated than the fat further removed from the external surface of the body. This fat near the surface acting as a protective covering may carry a more or less constant amount of the liquid, unsaturated acids. Mayer, Schaeffer, and Terroine, as quoted by Leathes (10), have termed the fat found in the organs and muscles which is largely in complex lipid combination and extracted with some difficulty as the "element constant," as distinguished from that in the adipose tissue which they term the "element variable." The element constant, frequently spoken of as metabolic fat, is more unsaturated than stored fat. It is reasonable to believe that a relatively higher proportion of the total body fat would consist of the surface fat and the metabolic fat in the young, thin animal than in the older, fat animal. It is, of course, impossible to preclude ingested linolic acid as the precursor of that found in the young animals, yet its presence in the amounts found may be the result of synthesis to meet certain demands of the tissues.

The total saturated acids were lowest in the first four animals. The increase in per cent following these was not only small but without uniformity. Indeed it is unusual to find such a low saturated acid content in the well fattened, mature animals. The carcass grading has shown an unusually hard adipose tissue, which at first sight is difficult to reconcile with the relatively low content of saturated acids. The absence of an appreciable amount of linolic acid would seem to be an important factor. The oleic acid on the other hand must be so combined with the palmitic and stearic acids into mixed glycerides as to lose much of its softening properties.

Four samples were chosen for fractionation of the saturated acids in order to determine the proportions of stearic, palmitic, and myristic acids occurring in the body fat. Saturated acid fractions of the fat from Hogs 5, 7, 11, and 13 were prepared, the methyl esters formed, and fractionally distilled under low pressure. After determining the saponification numbers on these fractions

the acids were identified and the proportions of each calculated. The results of this rather laborious method are given in Table V. Myristic acid constituted 0.7 to 1.1 per cent of the fat. Palmitic acid predominated among the three saturated acids present although the quantity was less than half that of oleic acid. Stearic acid on the other hand was comparatively low. In Hogs 5 and 7 it was approximately 33 per cent, while in Hogs 11 and 13 it approached to 50 per cent that of palmitic acid.

These results of fatty acid separations, reflecting as they do the results of fat anabolism in the hog when fed on a ration low in fat, show through their changes from the young suckling to the grown fattened animal some shifting in the type of fat deposited. They are interpreted as representing a close approximation of the normal body fat of hogs when the influence of ingested fat is at a minimum and other factors of nutrition are normal.

SUMMARY.

The changes in the quantity and composition of the body fat of hogs at successive intervals of growth and reared on a ration low in fat were found to be as follows:

1. The animals synthesized and stored fat at a normal rate. The lack of fat in the ration did not appear to exert material influence on the degree of fatness.

2. Hard, saturated fat was formed even in the young pigs. A gradual increase in saturation occurred up to a weight of approximately 100 pounds, above which extremely hard body fat was produced.

3. The principal fatty acids found in the fat were oleic, palmitic, and stearic. Others which occurred in small amounts were linolic, myristic, and arachidonic.

4. The principal change occurred in the linolic acid content. From a maximum content in the suckling pigs a steady decrease occurred up to a weight of 170 pounds. This change appeared to account for the increase in saturation of the fat which accompanied increase in weight.

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THE STABILITY OF VITAMIN G AS MEASURED BY ITS GROWTH-STIMULATING EFFECT.*

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INTRODUCTION.

In recent years it has been found that the original water-soluble vitamin of McCollum and Davis is composed of at least two essential substances (1–12). The earliest work concerning the two factors indicated that the so called growth factor was more resistant to destruction or inactivation by heat than the antineuritic factor.

During the course of an investigation in which we were attempting to concentrate and purify this supposedly stable factor, numerous irregularities and reverses were encountered. Our concentrate was often found much less potent per unit weight after a given treatment than it had been previous to this treatment. These discrepancies could not be explained without assuming that a destruction or an inactivation had taken place during the treatment.

In 1918 McCollum and Simmonds (13) reported that the dietary factor, vitamin B, was resistant to the action of nitrous acid, its activity being determined by its power to restore growth in rats upon a diet devoid of this factor. These investigators observed the growth of their experimental animals during a period of 2 weeks. Levene and van der Hoeven (14) reported similar results from animals which had been under their observation for only a few days. Later, however, Levene (15) reported that the heat-stable factor of the vitamin B complex was destroyed by rigorous treatment with nitrous acid. Emmett and Luros (1) reported that both

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yeast and unmilled rice retained their growth-promoting power for rats even after 6 hours heating at 120°, although the antineuritic effect for pigeons was lost after 2 or 3 hours of such heating. Smith and Hendrick (6) and Goldberger and associates (7) reported favorable results from feeding yeast which had been autoclaved 6 hours at 15 pounds pressure. Hassan and Drummond (16) reported an acceleration of the growth of rats by feeding an alkaline extract of yeast and concluded that this factor was not destroyed by hot alkali. Hogan and Hunter (12) obtained results indicating that this growth-stimulating substance was destroyed by irradiation for 10 hours with a mercury arc light at a distance of 20 cm.

Such was the available information concerning the stability of this factor when the present investigation was started. During the course of the investigation, however, several publications have appeared which throw further light upon this subject. Chick and Roscoe (17) found that ultraviolet light exerted a destructive action on both factors of the vitamin B complex, the growth factor being destroyed more quickly than the antineuritic factor. Chick deaminized yeast extracts by the method described by Peters (18) and observed the growth of rats receiving these preparations for a period of 3 weeks; she concluded that there had been no destruction by such a treatment. Williams, Waterman, and Gurin (19) investigated the effect of autoclaving yeast at various H ion concentrations and showed that the pH of the material when autoclaved determined the stability of the growth substance as well as the antineuritic. Kennedy and Palmer (20) reported that ultra-violet irradiation cannot be relied upon to destroy the growth factor.

The purpose of this investigation was to determine the effect of heat, H ion concentration, oxidation, reduction, deamination, and ultra-violet irradiation upon the growth-promoting activity of vitamin G in yeast or yeast extract.

EXPERIMENTAL.

Method of Testing Preparations.

Young albino rats, 25 to 30 days of age, and averaging 45 to 50 gm. in weight, were placed in group cages of a type which prevented access to excreta. Fresh portions of the basal diet (Diet 2 B) were supplied daily. This diet consisted of washed casein 18 parts, salt mixture (No. 186[1]) 3.7, agar 2, corn-starch 69.3, cod liver oil 2, butter fat 5. After a 2 weeks depletion period on this diet, the rats were placed in individual cages. In addition to an

[1] Salt 186 is Salt Mixture 185 of McCollum and Simmonds (McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918)) with dibasic Ca phosphate substituted for the monobasic and 0.004 per cent of KI added.

adequate supply of the above ration, each animal received, as a source of antineuritic factor, 0.05 gm. of activated fullers' earth prepared from an extract of white corn by a method formerly noted (21). Previous tests had shown this quantity to be adequate. After the 2 weeks depletion period, the feeding of the material to be tested was begun. The various preparations were weighed and fed daily in quantities equivalent to 0.15 gm. of net solids from the original extract. Previous experiments had shown that this quantity was adequate to produce a gain of from 12 to 15 gm. per week in young albino rats receiving the above basal diet. The various preparations were tested in this manner for a period of 8 weeks and the rates of growths compared. Controls were also run in which the animals received a similar diet and treatment, except that they received none of the fractions to be tested. A daily record was kept of the amount of basal ration placed in each cage. Each animal was weighed once a week and the residual food, together with any that might have fallen to the bottom of the cage, removed from the cage, sieved, and reweighed.

Method of Preparation.

Both bakers' yeast and extracts from bakers' yeast were used in this investigation. All extracts used were prepared by extracting yeast which had previously been extracted with gasoline, with 51 per cent alcohol (by weight) at the rate of 10 liters per kilo of yeast. This extract was concentrated by vacuum distillation to a volume of about 500 ml. per kilo of yeast used. The concentrates were placed in a large separatory funnel and shaken with successive portions of ether to free them of fatty materials. The extracts were returned to the vacuum still and concentrated at a low temperature until all ether had been removed. The concentrate thus obtained was divided into aliquots, the several portions subjected to the reacting agencies, their pH adjusted to about that of the original concentrated extract, then concentrated at a low temperature to a thick sirupy consistency, and mixed with a known weight of dry corn-starch. They were then dried in an oven at a low temperature, ground, and passed through a 40 mesh sieve. An untreated aliquot was finished in a similar manner to serve as a control. The daily dosage was calculated on the basis of the net solids in the untreated controls. The yeast

preparations were dried when necessary, pulverized, and fed in equivalent daily dosages of net solids.

Studies with Heated, Autoclaved, Oxidized, and Reduced Fractions.

Preparation of Fractions.—In order to test the effect of heat upon the growth-promoting properties of yeast, three 200 gm. portions of bakers' yeast, which had previously been extracted with gasoline, dried, and sieved, were used. One of these portions was tested without further treatment and considered as the control. A second portion was spread in a thin layer upon an enamel pan and heated in an electric oven for 4 hours at 120°. The third portion was placed in a large evaporating dish, moistened with distilled water, and autoclaved for 4 hours at 20 pounds of pressure. It was then removed from the autoclave, dried at a low temperature, and ground. Each of these portions was fed at the daily rate of 0.15 gm. per rat.

The second half of the concentrated yeast extract used in Series 230 (see Table III) was also divided into four equal aliquots and used in this phase of the study. Two of these portions were placed in separate flasks which in turn were placed upon a hot water bath maintained at a temperature of 85–90°. When the contents of these flasks had reached the temperature of the water bath, a stream of oxygen was passed through one solution, while a stream of hydrogen sulfide was similarly passed through the other. The source of oxygen was a commercial cylinder, while that of the hydrogen sulfide was a Kipp generator. The rate of the flow of the two gases was sufficient to keep each solution well agitated. This operation was continued for 4 hours with the temperature of the water bath maintained as constant as possible. When this treatment had been completed, these two fractions were concentrated under a vacuum, mixed with starch, and finished in the usual manner. The two remaining aliquots were placed in similar flasks, their pH adjusted to 2.9 and 10.1 respectively, and autoclaved for 4 hours at 20 pounds of pressure. When this operation had been completed, the pH of the two autoclaved aliquots was adjusted as close as practical to its original pH, and the two fractions further concentrated under a vacuum. They were then finished in the same manner as the above.

Results.—The results obtained by feeding the above fractions

are presented in Table I.   Both dry heating and autoclaving of yeast resulted in a marked decrease in the growth-stimulating activity of that substance.   This decrease amounted to about 20 per cent in each case.

The bubbling of H_2S through a hot yeast concentrate, under the conditions specified, showed no destructive action upon this

TABLE I.

Gain in Weight and Food Consumed by Rats Receiving Heated, Oxidized, and Reduced Preparations (0.15 Gm. of Net Solids per Rat Daily) during a Test Period of 8 Weeks.

Fraction No.	Treatment of fraction.	Average initial weight of rat.	Average gain per rat.	Total feed consumed per rat.
		gm.	*gm.*	*gm.*
232	Gasoline-extracted yeast.	64.0	100.8	492.9
239	" " heated 4 hrs. in oven at 120°.	52.0	76.5	409.4
240	Gasoline-extracted yeast moistened with H_2O and autoclaved 4 hrs. at 20 lbs.	53.3	80.8	424.4
230 A	Extract from 0.5 kilo yeast, concentrated and taken up on starch.	55.8	135.0	540.7
230 B	Same as Fraction 230 A, but reduced with H_2S 4 hrs. before taking up on starch.	55.5	140.3	532.5
230 C	Same as 230 A, but O_2 bubbled through warm concentrate 4 hrs. before taking up on starch.	53.5	120.5	503.0
230 G	Same as 230 A, but made acidic with HCl and autoclaved 4 hrs. at 15 lbs.	53.5	76.3	453.7
230 H	Same as 230 A, but made alkaline with NaOH and autoclaved 4 hrs. at 15 lbs.	53.0	27.0	282.0
Control.	No source of vitamin G added.	52.8	15.1	277.0

factor.   Whether or not this substance is destroyed by other reducing agents is yet to be determined.   The bubbling of molecular oxygen through a hot concentrated extract of yeast showed only a slight destructive action.   It hardly seems probable that this agency can be responsible for the destruction of vitamin G.

The effect of atomic oxygen upon this substance, however, might be quite different from that of molecular oxygen.

The destruction of vitamin G in yeast extracts by autoclaving apparently depends upon the pH of the extract when autoclaved as shown by Williams and associates (19). Autoclaving of alkaline extracts results in a greater destruction than a similar treatment to an acidic extract.

Studies with Deaminized Fractions.

Preparation of Fractions.—In the study of the effect of deamination upon the stability of the growth-stimulating substance, four series of fractions were prepared and tested. In the first (Series 229) the concentrated extract from 2 kilos of yeast was used. This concentrate was diluted to 1000 ml. in a volumetric flask, and 250 ml. were removed as a control. The remaining 750 ml. were placed in a 2 liter round bottom flask and subjected to the deaminizing process. This was accomplished by passing the gases, liberated from a saturated solution of sodium nitrite by concentrated sulfuric acid, through the concentrated yeast extract. The rate of flow of sulfuric acid was so regulated as to give a steady flow of bubbles through the solution. After this process had continued for 3.5 hours, one-third of the contents of the flask was removed; the process was continued for 4 additional hours, when another third (one-half of the remainder) was removed; the remainder was treated for 2.5 hours longer. One-tenth of each of the four aliquots was taken for amino nitrogen determination. The pH of each of the three deaminized aliquots was adjusted as close as practical to that of the control, and the four were further concentrated under a vacuum, mixed with corn-starch, dried at a low temperature, pulverized, and sieved. The amino nitrogen content of the preserved samples was determined by the Van Slyke method, and the total amount of this substance in each of the four preparations calculated.

The data obtained by feeding the above series of preparations showed that a marked destruction had resulted from the treatment. Consequently, we took the concentrated extract from 800 gm. of yeast, divided it into two equal portions, and subjected one-half to the above treatment, while the other half was retained as a

control. This time the deaminizing reaction was continued for 15 hours, and the two fractions completed by the same procedure as was used in the first series (No. 229). The results from feeding these preparations led to a third series (No. 257) consisting of three fractions. Each of these fractions represented the concentrated extract from 0.5 kilo of yeast. One-third of the concentrate was retained as a control, and another was deaminized for 15 hours by the above method. Previous experiments with this method had shown that considerable heat was generated during the reaction in the flask containing the concentrate, especially during the early part of the process. To avoid this factor, the flask containing this aliquot was immersed in an ice bath and continuously agitated by an electric stirrer during the 15 hours. A third portion was deaminized by the method of Peters (18). After the two aliquots had been deaminized, a portion of each as well as of the control was taken for amino nitrogen determination. The hydrogen ion concentration of the two deaminized fractions was adjusted to within 0.1 pH of that of the control, the three extracts further concentrated under a vacuum, and finished up in the usual manner.

Since the results obtained by the feeding of the third series of fractions (Series 257) showed that a decrease in the amino nitrogen content of fractions was not always followed by a corresponding decrease in the growth-stimulating property, a fourth series (No. 259) of fractions was prepared to determine the effect of more complete deamination upon this property. Series 259 was made with the concentrated extract from 1.5 kilos of yeast. One-third of this concentrated extract was treated in the usual manner to serve as a control. To the remaining two-thirds, 5 per cent concentrated HCl was added and the mixture refluxed on a sand bath for 4 hours. The hydrolysate was cooled and divided into two equal portions. One portion was brought approximately to its original pH, concentrated, mixed with starch, and finished in the usual manner. The third portion was deaminized by adding solid sodium nitrite from time to time until a total of 37.5 gm. had been added to the already acidified solution. After standing for 24 hours, it was then finished in the usual manner. Samples of the three fractions were taken for amino nitrogen determination previous to the final concentration.

Results.—The results obtained by feeding the above fractions

TABLE II.

Gain in Weight and Food Consumed by Rats Receiving Deaminized Preparations (0.15 Gm. of Net Solids per Rat Daily) during a Test Period of 8 Weeks.

Fraction No.	Treatment of fraction.	NH$_2$-N left after treatment.	Average initial weight of rat.	Average gain per rat.	Total feed consumed per rat.
		per cent	*gm.*	*gm.*	*gm.*
229 B	Extract from 0.5 kilo yeast, concentrated and mixed with starch.	100.0	53.5	99.8	480.0
229 A	Extract from 0.5 kilo yeast, concentrated, deaminized 3.5 hrs.	38.3	50.0	53.0	312.4
229 C	Same as Fraction 229 A but deaminized 7.5 hrs.	13.9	51.5	31.0	304.4
229 D	Same as 229 A but deaminized 10 hrs.	9.5	51.5	31.0	334.0
250 A	Extract from 0.4 kilo yeast, concentrated and mixed with starch.	100.0	51.5	119.0	555.0
250 B	Same as 250 A but deaminized 15 hrs.	11.3	54.5	50.5	380.5
257 A	Extract from 0.5 kilo yeast, concentrated and mixed with starch.	100.0	47.5	97.5	471.0
257 B	Same as 257 A but deaminized 15 hrs.	27.6	46.5	86.0	460.0
257 D	Same as 257 A but deaminized by Peters' method.	19.5	47.0	86.5	467.5
259 A	Extract from 0.5 kilo yeast, concentrated and mixed with starch.	100.0	51.5	110.0	434.0
259 B	Same as 259 A but hydrolyzed before mixing with starch.	100.0	52.5	129.0*	525.0
259 C	Same as 259 B but deaminized before mixing with starch.	2.5	54.0	110.0	493.0
Control.	No source of vitamin G added.		52.8	15.1	277.0

* The greater growth on this fraction may be attributed to the sex of the animals used. This fraction was fed only to male rats, while all other fractions were fed to an equal number of each sex. The gain made by these rats compares well with that made by male rats receiving the other fractions of this series.

are presented in Table II. The data obtained from Series 229 show that a decrease in the amino nitrogen was accompanied by a decrease in growth-stimulating qualities; these decreases, however, were not proportional to each other. More than 60 per cent of the amino nitrogen was removed during the first 3.5 hours of treatment, but the growth-producing effect was reduced less than half. Continuing this treatment for 4 additional hours resulted in further loss of amino nitrogen but its growth effect remained practically unchanged. Although further amino nitrogen was removed by continuing the process from 7.5 to 10 hours, the effect upon growth remained unchanged. This point was difficult to explain at the time, but further data seem to throw some light upon it and may offer a possible explanation. It was remembered that the above mode of deamination was continued for 7.5 hours the 1st day. During this time, as formerly mentioned, the solution became quite warm and continued so throughout the day. The following night it became cool and during the 2.5 hours treatment the next day no indication of heating was observed. It is possible that the destruction was due to the heating effect and not to a direct loss of amino nitrogen.

The results from the two members of the second series (No. 250) are in accord with those of Series 229. A decrease of 89 per cent in amino nitrogen was followed by only a 58 per cent loss in the growth effect. Results obtained on Fraction 257 D add further evidence that the greater portion of the active substance destroyed by this method resulted from side reaction, associated with heating phenomena, and not from the loss of amino nitrogen. The third series (No. 257) clearly shows that the decease in growth-stimulating qualities cannot be due to the loss of amino nitrogen.

Fraction 259 C gave equally as good growth as the untreated fraction (No. 259 A), although 97.5 per cent of its amino nitrogen had been removed. A point to be noted in this connection is the fact that Fractions 259 B and 259 C were refluxed in a 5 per cent hydrochloric acid solution for 4 hours without any apparent effect upon their growth-stimulating qualities. The results obtained on feeding the fourth series (No. 259) add further proof that deamination, in itself, is not responsible for the decrease in growth-stimulating properties sometimes encountered after this treatment.

Studies with Irradiated Fractions.

Peparation of Fractions.—The irradiated fractions used consisted of two series. The first, or Series 230, consisted of four preparations. One-half of the concentrated extract from 4 kilos of yeast, prepared in the above manner, was diluted to 800 ml. and divided into four equal aliquots. One aliquot was further concentrated to a small volume by vacuum distillation, mixed with 100 gm. of dry corn-starch, and dried; this fraction served as a positive control. The pH of two of the aliquots was adjusted to 2.9 and 10.1, respectively, with HCl and NaOH. These with the fourth aliquot, which had a pH of 4.7, were placed in large crystallization dishes and exposed to rays of a mercury arc (Cooper Hewitt) for 20 hours at a distance of 30 cm. The depth of these concentrates in the containers did not exceed 1.5 cm. at the beginning of the irradiation and diminished but slightly during this period. The containing vessel was shaken at hourly intervals so as to bring new material to the exposed surface. After irradiation, the three aliquots were adjusted to pH 4.8, 5.0, and 4.7, concentrated under a vacuum, mixed with starch, and treated in a manner similar to the control aliquot.

The results obtained with Series 230 indicated that the alkali had greatly increased the destructive action on the growth substance. What part of this destruction could be attributed to the alkali alone could not be determined from the data. Consequently, a second series (No. 253) was prepared. The concentrate from 3 kilos of yeast was diluted to 1200 ml. with a small quantity of water. It was then divided into three equal aliquots of 400 ml. each. The pH of the three aliquots was adjusted to 3.2, 6.5, and 8.2, respectively. Each of these three aliquots was again divided into two equal parts, making a total of six, each representing the extract from 0.5 kilo of yeast. Three of these aliquots, each of a different pH, were placed in the crystallization dishes and irradiated in a manner similar to Series 230. In the meantime, the three remaining aliquots were stored in a dark cabinet. When the irradiation had been completed, the pH of all six aliquots was brought within the limits of 6.4 to 6.6. Each was then concentrated under a vacuum, mixed with 100 gm. of dry starch, and finished in a manner similar to those of Series 230.

Results.—The results obtained from feeding the above fractions

are shown in Table III. From the data presented in Table III
it is evident that these yeast extracts became less effective growth
producers as a result of this treatment. The first three (Series 230)
preparations show a considerable variation in growth-stimulating

TABLE III.

Gain in Weight and Food Consumed by Rats Receiving Irradiated Preparations
(0.15 Gm. of Net Solids per Rat Daily) during a Test Period of 8 Weeks.

Fraction No.	Treatment of fraction.	Average initial weight of rat.	Average gain per rat.	Total feed consumed per rat.
		gm.	*gm.*	*gm.*
230 A	Extract from 0.5 kilo of yeast, concentrated and mixed with corn-starch.	55.8	135.0	540.7
230 F	Same as Fraction 230 A but irradiated 20 hrs.	53.0	112.3	452.0
230 D	Same as 230 A but acidified with HCl and irradiated 20 hrs.	58.5	130.5	521.4
230 E	Same as 230 A but made alkaline with NaOH and irradiated 20 hrs.	55.0	54.0	358.0
253 A	Extract from 0.5 kilo yeast, concentrated, stored in dark, mixed with starch; pH 6.5.	47.5	112.0	512.0
253 B	Same as 253 A irradiated 20 hrs. instead of storing in dark.	46.0	79.0	488.0
253 C	Same as 253 B but brought to pH 3.2 before irradiating.	48.5	87.5	474.0
253 E	Same as 253 C but stored in dark instead of irradiating.	50.3	109.2	491.0
253 D	Same as 253 B but brought to pH 8.2 before irradiating.	47.0	75.5	446.0
253 F	Same as 253 D but stored in dark instead of irradiating.	53.0	100.5	508.0
Control.	No source of vitamin G added.	52.8	15.1	277.0

qualities.   This loss of activity ranged from about 3 per cent in the
case of the most acidic preparation to 60 per cent in the case of the
most alkaline one.

The results obtained with Series 253 agree well with those
obtained with Series 230.   Due to the wider range of hydrogen con-

centration used in Series 230, a greater variation between the activity of the members of this series is found. The maximum variation in Series 253 represents a loss of about 35 per cent. About 10 per cent of this amount is due to the pH of the concentrate or other factors and the remainder to irradiation. The minimum amount of destruction occurred in the most acidic member of the series; the decrease amounted to about 23 per cent, of which about 20 per cent could be attributed to irradiation. From the data presented in Table III it is evident that yeast extracts became less effective as growth producers as a result of the irradiation under the described experimental conditions. Moreover, it appears that while hydrogen ion concentration may not profoundly affect the destructive action of ultra-violet light upon vitamin G, it does greatly affect the stability of this factor in aqueous solution.

The magnitude of the destruction of this factor by irradiation as observed in this investigation does not agree entirely with that obtained by others (12). We are unable to explain this difference, but there is a possibility that it may be at least partially due to the variation in activity of rays emitted by the different mercury arcs.

SUMMARY.

1. Yeast and yeast extracts, autoclaved for 4 hours at 20 pounds, lost some of their growth-stimulating effect.

2. Dry yeast heated for 4 hours at 120° showed a similar loss of activity.

3. The passing of gaseous oxygen through a hot concentrated yeast extract for 4 hours had very little effect upon its growth-stimulating qualities.

4. The passing of H_2S gas through a hot concentrated yeast extract for 4 hours had no effect upon its growth qualities.

5. Whether partial deamination of yeast extracts decreased their growth-promoting effect seemed to depend upon side reactions, rather than upon deamination of the free amino groups.

6. Yeast extracts concentrated to a thick sirupy consistency and irradiated at a distance of 20 cm. from a mercury arc showed some decrease in growth-stimulating effect. The total destruction was greater in alkaline than in acid so`ution, the result apparently being the combined effect of the separate action of alkali and irradiation.

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THE RELATIVE AMOUNTS OF IODINE-ABSORBING (REDUCING) MATERIAL IN VARIOUS PLANTS.

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The studies which we have made suggest that certain plants contain antigoitrous substances (1–3) other than iodine. This antigoitrous effect seems to be proportional to the amount of iodine-absorbing material in the plant. Evidence has been published (4) which indicates that the iodine-absorbing material contains a factor which inhibits thyroid hyperplasia and that it may be identical with the hexuronic acid which Szent-Györgyi (5) has isolated from cabbage, orange, and suprarenal cortex, and which appears to be present also, in high concentration, in corpus luteum.

In the course of our investigations of these iodine-absorbing substances, it was necessary to survey available plants for the best source of this material, and the data thus far obtained are given in the present paper.

Method.

As some of the reducing substances—particularly those with which this paper deals—are destroyed by exposure to air and by oxidizing enzymes, it is necessary to protect them. The enzymes can be inhibited by cyanides, by lowering the pH, or they may be destroyed by steaming. Since we were interested primarily in the physiological effects of these reducing substances, we have in all these studies destroyed the enzymes by steaming (97–100°) for 30 to 35 minutes in a heavily tinned copper sieve in a surgical utensil sterilizer.

A small amount of the steamed material is rapidly ground in a mortar and 10 gm. of the pulp are put in a beaker with about

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50 cc. of distilled water. 1 cc. each of hydrochloric acid and starch-iodine solution are added and the mixture titrated with 0.01 N iodine, the end-point being a faint bluish coloration that holds for 30 seconds. The time required for the procedure after steaming is approximately 5 minutes. Tough or woody leaves, stems, and roots are cut finely with scissors before grinding. Press juice from the steamed material is titrated in the same way as for the whole hashed material. This method of titration was chosen because it provided a simple, rapid, and relatively constant means of obtaining comparable data from a wide range of plants. The data which we have obtained from the plant tissues so far surveyed by the above method are summarized in Table I.

In Table I we have indicated the number of specimens examined and the highest and lowest iodine-absorbing values obtained. We have also indicated whether the material was obtained fresh from the field or garden, or purchased in the market. This is important because the iodine-absorbing material begins to disappear within a few hours after the plant is gathered. Storage in an ice box greatly lessens the rate of its disappearance. It is therefore necessary that estimations of the iodine-absorbing material be made while the plants are in the freshest possible condition.

It is of interest that all of the ordinary vegetables examined had comparatively low iodine-absorbing values. In general, the leaves contain more of the material than the roots (beet, carrot). It was first thought that those plants which grew in the early spring (daffodil, skunk-cabbage) had higher iodine-absorbing values than those plants requiring higher temperatures for growth, but there are exceptions as in the case of the tulip. Alfalfa, the clovers, and lawn grass are relatively high and could easily serve as a source of the iodine-absorbing material. The plants which we have found to contain the greatest amount of iodine-absorbing material are the leaves of skunk-cabbage, iris, gladiolus, and sugar maple.

Seasonal Variations.—In practically all the plants examined, it was found that the iodine-absorbing material is present in greatest concentration in the young growing portions of the leaves and stalk, and that there is a gradual, and sometimes rapid, fall during maturation. The leaf of the sugar maple has shown the most rapid decrease of any plant we have examined. The young unfolding leaves on May 1 absorbed 40 cc. of 0.01 N iodine per 10 gm.,

TABLE I.

Iodine-Absorbing Material in Plant Pulp.

Plant.	Where obtained.	No. of specimens examined.	0.01 N I absorbed by 10 gm. steamed pulp.	
			High.	Low.
			cc.	*cc.*
Alfalfa (*Medicago sativa*)................	Fresh.	15	20.6	5.4
Asparagus (tips) (*Asparagus officinalis*)..	"	2	13.0	9.5
Beet root (*Beta vulgaris*).................	"	2	2.2	0.7
" leaves " "	"	3	2.3	0.6
Cabbage (*Brassica oleracea*)..............	Market.	677	5.4	2.6
Carrot root (*Daucus carota*)..............	Fresh.	3	4.3	1.0
Clover (*Trifolium pratense*)..............	"	3	17.6	17.5
Castor bean (leaves) (*Ricinus communis*)..	"	1	4.7	
Chard (*Beta cicla*).......................	"	5	3.7	0.6
Daffodil (leaves) (*Narcissus pseudo-narcissus*)...........................	"	9	16.5	5.2
Gladiolus................................	"	4	34.5	17.5
Horseradish (leaves) (*Roripa armoracia*).	"	1	17.7	
Hyacinth (leaves) (*Hyacinthus orientalis*)................................	"	2	19.6	18.0
Iris (*Iris sibirica*).......................	"	5	8.6	6.0
" (" *germanica*).....................	"	27	48.0	16.0
Kohlrabi (*Brassica oleracea caulorapa*)...	"	3	8.8	4.3
Lawn grass..............................	"	5	18.0	11.0
Leek (*Allium porrum*)...................	"	2	6.0	4.0
Lettuce (*Lactuca sativa*).................	"	7	1.7	0.5
Maple (leaves) (*Acer saccharinum*).......	"	3	40.0	2.2
Nasturtium (*Tropæolum majus*)..........	"	2	9.0	8.0
Onion (bulb) (*Allium cepa*)..............	Market.	1	0.9	
Potato (leaves) (*Solanum tuberosum*)....	Fresh.	4	4.3	0.8
Radish " (*Raphanus sativus*)......	"	4	7.5	6.0
Rutabaga (*Brassica campestris rutabaga*).	Market.	1	4.6	
Skunk-cabbage (1929) (*Symplocarpus fœtidus*)............................	Fresh.	15	26.2	1.8
Skunk-cabbage (1930)..................	"	99	23.7	0.8
Spinach (*Spinacia oleracea*).............	Market.	2	3.5	2.6
Sunflower (leaves) (*Helianthus annuus*).	Fresh.	1	4.0	
Tomato (leaves) (*Lycopersicon lycopersicum*).............................	"	6	2.4	1.0
Tulip (leaves) (*Tulipa gesneriana*).......	"	11	3.2	0.8

while on May 7 the fully formed leaves from the same branch had dropped to 5.4 cc. per 10 gm.

In order to demonstrate in detail the gradual decline in iodine-absorbing material with age, we have given in Table II the averages of the weekly readings for alfalfa, daffodil, iris, skunk-cabbage, and tulip.

We would again point out that only green, freshly gathered leaves were used and all of the samples of each of the species tabulated were taken from the same field or garden. In the case of alfalfa, it is seen that the iodine-absorbing value remained high from April 8 to May 5, and during the next week (in blossom) it dropped abruptly to approximately one-third its former value as was the case with maple leaves. Daffodil, iris, skunk-cabbage, and tulip have shown a more gradual falling off in their iodine-absorbing material over a period of from 2 to 4 months. It is striking to observe a drop to one-fortieth or one-fiftieth the amount of iodine-absorbing material in skunk-cabbage leaves without any change in their external appearance.

There is also some evidence of *annual variation*. Skunk-cabbage remained high during the month of May, 1929 (a wet, cool month) while in May, 1930 (a dry, warm month) there was a rapid falling off in the iodine-absorbing material.

Cabbage also shows seasonal variations in the amount of reducing substances as is indicated in the following averages (for the year 1929) of the total monthly readings expressed in cc. of 0.01 N iodine per 10 gm. of plant pulp: April 5.4, July 4.7, September 3.5, October 3.5, November 2.8, and December 2.6.

Variations in Amount of Iodine-Absorbing Material in Different Parts of the Plant.—As already pointed out, the growing tips have the highest percentage of iodine-absorbing material and the more mature portions have the least. This is illustrated in the case of alfalfa. Alfalfa stalks 60.0 cm. long were gathered and steamed within 2 hours. The steamed stalks were divided into pieces approximately 15 cm. long. The lowest portion had an iodine-absorbing value of 3.4 cc. of 0.01 N iodine per 10 gm.; the second portion, 6.6 cc.; the third portion, 13.7 cc.; the top portion, 16.3 cc.

Effect of Storage and Drying.—Plant leaves when stored exposed to air, gradually lose their iodine-absorbing power. Thus, skunk-cabbage leaves brought to the laboratory on April 22 and stored

TABLE II.

Iodine-Absorbing Material in Relation to Physiological Cycle.

Plant.	No. of specimens examined.	Cc. 0.01 N I absorbed by 10 gm.																		
		Apr. 1-7.	Apr. 8-14.	Apr. 15-21.	Apr. 22-28.	Apr. 29-May 5.	May 6-12.	May 13-19.	May 20-26.	May 27-June 2.	June 3-9.	June 10-16.	June 17-23.	June 24-30.	July 1-7.	July 8-15.	July 16-22.	July 23-29.	July 30-Aug. 5.	Aug. 6-13.
Alfalfa	10		17.2		20.6	18.6	6.5													
Daffodil	8		16.5	16.1		15.6	15.4	6.5	6.4		6.0	5.2								
Iris	26	47.0	45.1	47.0	30.2	48.0		37.3		45.7	38.7	29.4		32.0		32.0		27.4	21.0	19.0
Skunk-cabbage, 1929	15	23.7	17.8	25.0	22.0	21.2	16.0	19.5		23.0						2.0				
" 1930	99							12.0	8.3	6.7	6.1	2.9	5.7	4.8	2.3	2.0	0.8	2.3	2.5	
Tulip	11	1.1	2.3	1.7	2.3	2.5	2.5	3.2	0.8		1.0									

overnight in bags on the roof, dropped from 22.0 units[1] per 10 gm. to 15.5 units.   When stored in an ice box at temperatures varying between 5–10°, deterioration occurs, but more slowly.   A sample of alfalfa brought to the laboratory on April 30 titrated 20.0 units per 10 gm.   This was stored in an ice box and on May 2 the value had dropped to 16.0 units and on May 5 to 13.0 units.   These observations are similar to the losses of the iodine-absorbing material in ox suprarenal glands reported by Szent-Györgyi.

Drying in air causes a very rapid loss of the iodine-absorbing material during the first 2 or 3 days; afterwards (in the case of alfalfa) the loss is very gradual during the next 3 or 4 months.

Comment.

It is probable that the iodine-absorbing material of plants includes a number of substances perhaps wholly unrelated chemically and physiologically.   Two of these substances are glutathione, isolated by Hopkins (6), and the hexuronic acid isolated by Szent-Györgyi, and, in the higher plants, glutathione makes up only a small portion of the total.

The physiological importance of these reducing substances is still unknown.   Many additional facts support the original view of Hopkins that they belong to biological oxidation systems. They are present in greatest concentration in the rapidly growing parts of the plant, which suggests that they take part in the metabolism of growth and synthetic processes.   They gradually disappear from the older portions of the plant and from the plant as a whole during maturity.   Further, we have reported evidence which indicates that in animals, these reducing substances exert an antigoitrous influence on the thyroid gland as shown by their ability to involute thyroid hyperplasia when fed by mouth or injected intraperitoneally.   This observation would suggest that they have a sparing action on the thyroid, possibly by providing another means of promoting or regulating tissue oxidations.

Nothing is known of the fate of these reducing substances in the plant.   They are not stored as such in the rootstocks or rhizomes of the two plants whose leaves contained the greatest concentration—skunk-cabbage and iris—either during the active growth or

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[1] Units represent cc. of 0.01 N iodine.

after maturation.   We have repeatedly found iris leaves which absorbed more than 40 units per 10 gm., while the rhizomes absorbed about 3 units per 10 gm.   Examinations of the rootstocks of skunk-cabbage during the height of activity and during their resting period likewise have shown no essential differences.   On April 20 during the height of growth, the leaves absorbed 26 units of iodine per 10 gm., while the rootstock absorbed 3.7 units per 10 gm.   Rootstocks obtained on November 22 absorbed 4.4 units per 10 gm. and most of this iodine-absorbing material appeared to be glutathione.

SUMMARY.

The relative amount of iodine-absorbing (reducing) material in plants is highly variable in different species and in the same species at different stages of its growth and maturation.   The reducing substances are present in greatest concentration in the rapidly growing portions of the plant and fall off gradually or abruptly during maturation.   There is some evidence of annual variations dependent upon climatic conditions.

Of the plants included in this survey, skunk-cabbage and iris leaves have given high values, while garden vegetables in general have shown low values.   Clover, alfalfa, and certain grasses also contain high concentrations during the period of rapid growth.

The iodine-absorbing material decreases slowly with storage and rapidly during drying.

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THE EFFECT OF CRUDE FIBER ON CALCIUM AND PHOSPHORUS RETENTION.*

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It is still undecided whether the calcium and phosphorus of vegetables are as well utilized by the animal organism as those of milk. Rose (15) and McLaughlin (12) have found the calcium from vegetables to be adequate in meeting the maintenance needs of man. Their subjects remained in calcium equilibrium when most of the milk of the diet was replaced by carrots in Rose's investigation, and by spinach in McLaughlin's. In similar experiments Blatherwick and Long (1) found that both calcium and phosphorus from vegetables sufficed to change negative to positive balances. On the other hand, Boas (3) showed that adding winter spinach to the diet of young rats did not raise the level of either calcium or phosphorus retention. McClugage and Mendel (10), working with adult dogs, and Sherman and Hawley (17), in their studies on children, found that vegetables were not as good a source of calcium as milk. In seeking a cause for this difference in mineral metabolism, the presence of crude fiber in vegetables suggests itself as a possibility worthy of investigation. In addition, if the vegetable fiber is found to influence the utilization of minerals, the question arises: How does it act? Does it increase peristalsis, and thus hasten the passage of the food through the digestive tract without allowing sufficient time for absorption to take place, or, does the fiber actually combine with the calcium and phosphorus of the food in such a way as to prevent, in large measure, their utilization by the tissues?

The present study was undertaken (1) to observe the effect of crude fiber in the diet on the retention of calcium and phosphorus by the body, and (2) to note whether the availability of these elements differed when the source was raw or cooked spinach.

<div align="center">EXPERIMENTAL.</div>

General Procedure.—Two metabolism studies were conducted on growing albino rats. The experimental animals were from stock which had been reared in this laboratory and fed Sherman's normal Diet B (16), with a small amount of lettuce or carrots added several times a week. After weaning, the young rats received this same stock diet. During the actual balance experiments, the animals were kept in metabolism cages essentially of the type described by Still and Koch (22).

Each experiment was of 1 week's duration, preceded by a preliminary period of 4 or 5 days during which the animals became adjusted to the metabolism cages and to the new diets. A litter of eight rats served as subjects in Experiment A; two rats were placed in each cage and received one of the experimental diets. The rats were 63 days old at the beginning of the experiment, and ranged in weight from 122 to 171 gm. For Experiment B twelve rats from two litters were placed in metabolism cages, three in a cage, and treated in a similar manner. The rats of this group were a month younger than the others—that is, 33 days old—and weighed from 36 to 57 gm. each. Both sets of rats were healthy and appeared to be as active during the metabolism studies as normally.

Diet.—The basal diet contained white flour, dried whole milk, butter fat, yeast, cod liver oil, and spinach ash. In the spinach diets the ash was replaced by either raw or cooked dried spinach. In the cellulose diets ashless filter paper was added to the basal diet, in one case enough to make the crude fiber content of the diet equal to that of the spinach diets, in the other sufficient to make it 30 times as high. The quantities of these diets fed were planned to be adequate in caloric value, and in protein, vitamin, and mineral content for normal growth of the rats. The acid-base residue was kept constant, as a change in pH might have affected both calcium and phosphorus retention, and the path by which they were excreted (4, 19, 20). The composition of these diets is reported in Table I.

Before beginning the experiment, the dry ingredients of each diet for the entire period were thoroughly mixed and sifted. A portion of this mixture was weighed out daily, the butter fat and cod liver oil added to it, and moistened with enough distilled water to make a paste. An excess over the probable food intake, based on the actual intake of the previous day, was placed in each cage. The residue was weighed on the following day and the food consumption computed.

TABLE I.

Composition of Diets.

	Diet 1.	Diet 2.	Diet 3.	Diet 4.	Diet 5.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
White flour....................	56.1	52.0	52.0	55.7	48.1
Dried whole milk.............	28.0	28.0	28.0	28.0	28.0
Butter fat....................	10.0	10.0	10.0	10.0	10.0
Yeast........................	5.0	5.0	5.0	5.0	5.0
Dried raw spinach............		5.0			
" cooked spinach.........			5.0		
Spinach ash...................	0.9			0.9	0.9
Filter paper..................				0.4	8.0
Cod liver oil, 1 drop per rat per day on all diets.					
Calcium.*....................	0.448	0.421	0.455	0.410	0.365
Phosphorus.*.................	0.336	0.347	0.324	0.339	0.342
Crude fiber.*................	0.33	0.66	0.74	0.70	8.38

* Analysis of dry ingredients only.

Excreta.—The feces were collected from the wire screens beneath the floor of the cage three or four times during the day, weighed, treated with a mixture of 9 parts of alcohol and 1 part of concentrated hydrochloric acid, and dried to constant weight. The dried material was then ground, sifted through a 20-mesh sieve, and stored in a glass-stoppered bottle in a desiccator. After removal of the feces, the bottom of the cage, the wire screen, and the glass funnel were thoroughly rinsed with distilled water. The washings were collected in a beaker containing toluene and evaporated to a small volume on a steam bath. Finally, the composite urine for the entire period was acidified with concentrated hydrochloric acid and made up to a definite volume.

Chemical Analyses.—Determinations of the calcium content of the urine and of dry-ashed samples of food and feces were made according to McCrudden's volumetric method (11). Precautions were observed for maintaining the correct hydrogen ion concentration as described by Shohl (18). Phosphorus determinations on food and feces were made according to Neumann's directions (13) for wet ashing, and the official gravimetric molybdate-magnesia method as set forth by McCandless and Burton (9), the suggestions of Lundell and Hoffman (8) being observed. The phosphorus of the urine was determined by Briggs' modification of the Bell-Doisy

TABLE II.

Food Intake and Its Crude Fiber Content.

	Food consumed per cage during wk.	Food consumed per gm. of rat during wk.	Crude fiber ingested, total.	Crude fiber ingested per gm. of rat.	Food exclusive of crude fiber, per gm. of rat.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Experiment A (older rats).					
Low fiber, Diet 1..............	182.8	0.51	0.54	0.0015	0.51
Raw spinach, Diet 2...........	148.5	0.56	0.88	0.0033	0.56
Cooked spinach, Diet 3........	180.0	0.53	1.20	0.0035	0.53
Filter paper, Diet 4...........	170.4	0.55	1.07	0.0034	0.55
Experiment B (younger rats).					
Low fiber, Diet 1..............	142.9	0.85	0.42	0.0025	0.84
Raw spinach, Diet 2...........	127.7	0.87	0.76	0.0052	0.87
Cooked spinach, Diet 3........	121.1	0.85	0.81	0.0057	0.85
Filter paper, Diet 5...........	173.9	0.92	13.11	0.0697	0.85

colorimetric method (5), after checking this procedure against the gravimetric method. Crude fiber was determined by the method of the Association of Official Agricultural Chemists (14).

Results.

Quantities of Food and of Crude Fiber Consumed.—The food consumed during the experimental periods and the crude fiber of this food are recorded in Table II. The younger animals ate more per unit of body weight than the older ones. In both series of experiments, the rats on the spinach and filter paper diets ate slightly

more per gm. of body weight than the controls, particularly on Diet 5, where the roughage formed such a large percentage of the diet. When, however, the crude fiber is subtracted from the total intake, the actual food ingested is seen to have been about the same on the high filter paper diet as on the other diets in Experiment B. The crude fiber content of the spinach diets and of the first filter paper diet was approximately the same, whereas the high filter paper ration, Diet 5, contained 28 times as much crude fiber per gm. of rat as the control diet.

TABLE III.

Quantity of Feces Excreted.

	Low fiber, Diet 1.	Raw spinach, Diet 2.	Cooked spinach, Diet 3.	Filter paper, Diet 4.
	gm.	*gm.*	*gm.*	*gm.*
Experiment A.				
Feces, total for wk..................	11.1	6.9	10.0	10.0
" per gm. of rat................	0.031	0.026	0.029	0.032
Feces : food.........................	1:16	1:22	1:18	1:17
				Diet 5.
Experiment B.				
Feces, total for wk..................	5.0	7.9	8.5	19.3
" per gm. of rat................	0.030	0.054	0.060	0.103
Feces : food.........................	1:29	1:16	1:14	1:9

Weight of Feces.—In the experiment on the younger animals there is a definite relationship between the weight of the feces and the character of the diet (Table III). Here, on the spinach diets (crude fiber content, 0.7 per cent), the quantity of feces excreted was about twice as great per gm. of body weight as on the control diet (crude fiber content, 0.3 per cent), and on the filter paper diet (crude fiber content, 8.4 per cent) over 3 times as great. The ratio of feces excreted to food intake, moreover, seems to be directly related to the crude fiber content of the diet. On the low fiber diet the ratio was 1:29, on the spinach diets 1:16 and 1:14 respectively, and on the high fiber diet 1:9. With the older rats, on the other hand, there appears to be no correlation between diet and feces.

TABLE IV.

Calcium and Phosphorus Metabolism.

Experiment A.	Diet 1.	Diet 2.	Diet 3.	Diet 4.
	gm.	gm.	gm.	. gm.
Calcium metabolism.				
Intake, total....................	0.74	0.56	0.74	0.63
Excretion, total................	0.31	0.38	0.52	0.29
Feces.........................	0.28	0.35	0.50	0.25
Urine........................	0.03	0.03	0.02	0.04
Retention, total................	0.43	0.18	0.22	0.34
Intake per gm. of rat........	0.00207	0.00212	0.00218	0.00202
Excretion " " " "	0.00087	0.00144	0.00153	0.00093
Retention " " " "	0.00120	0.00068	0.00065	0.00109
Per cent of intake retained......	58	32	30	54
" " " total excretion in feces.........................	90	92	96	86
Phosphorus metabolism.				
Intake, total..	0.55	0.46	0.52	0.52
Excretion, total................	0.25	0.25	˙ 0.32	0.25
Feces.........................	0.12	0.04	0.08	0.13
Urine........................	0.13	0.21	0.24	0.12
Retention, total....	0.30	0.21	0.20	0.27
Intake per gm. of rat........	0.00154	0.00174	0.00153	0.00167
Excretion " " " "	0.00070	0.00095	0.00094	0.00080
Retention " " " "	0.00084	0.00079	0.00059	0.00087
Per cent of intake retained......	55	46	38	52
" " " total excretion in feces.........................	48	16	25	52
Ca:P of intake...................	1.3:1	1.2:1	1.4:1	1.2:1
" " retention.................	1.4:1	0.9:1	1.1:1	1.3:1

Experiment B.	Diet 1.	Diet 2.	Diet 3.	Diet 5.
Calcium metabolism.	gm.	gm.	gm.	gm.
Intake, total....................	0.58	0.48	0.50	0.57
Excretion, total..	0.04	0.28	0.26	0.07
Feces.........................	0.02	0.27	0.25	0.05
Urine........................	0.02	0.01	0.01	0.02
Retention, total................	0.54	0.20	0.24	0.50
Intake per gm. of rat........	0.00344	0.00328	0.00353	0.00303
Excretion " " " "	0.00024	0.00192	0.00183	0.00037
Retention " " " "	0.00320	0.00136	0.00170	0.00266

TABLE IV—*Concluded*.

Experiment B—*Continued*.	Diet 1.	Diet 2.	Diet 3.	Diet 5.
	gm.	*gm.*	*gm.*	*gm.*
Per cent of intake retained......	93	42	48	88
" " " total excretion in feces.........................	50	96	96	71
Phosphorus metabolism.				
Intake, total....................	0.43	0.40	0.35	0.54
Excretion, total..	0.07	0.12	0.12	0.11
Feces.........	0.06	0.07	0.08	0.10
Urine.......................	0.01	0.05	0.04	0.01
Retention, total................	0.36	0.28	0.23	0.43
Intake per gm. of rat........	0.00255	0.00274	0.00247	0.00287
Excretion " " " "	0.00041	0.00082	0.00085	0.00058
Retention " " " "	0.00214	0.00192	0.00162	0.00229
Per cent of intake retained......	84	70	66	80
" " " total excretion in feces.........................	86	58	67	91
Ca:P of intake	1.3:1	1.2:1	1.4:1	1.1:1
" " retention................	1.5:1	0.7:1	1.0:1	1.2:1

Calcium and Phosphorus Metabolism.

Intake.—The ratio of calcium to phosphorus in the food varied from 1.1:1.0 to 1.4:1.0 according to the composition of the diet; in all cases it was within the normal range (Table IV). In the spinach diets, about 10 per cent of the total calcium intake was derived from the calcium of the spinach, and about 13 per cent of the phosphorus from the spinach phosphorus. In general, there was no correlation between intake and output on the different diets in either calcium or phosphorus metabolism.

Retention.—The total amount of calcium retained by the older group of rats (Experiment A) was highest on the low crude fiber diet, intermediate on the filter paper diet, and lowest on the spinach diets. On Diet 1, 1.20 mg. of calcium per gm. of rat was retained during the experimental period, on Diets 2 and 3, 0.68 and 0.65 mg. respectively, and on Diet 4, 1.09 mg. Thus while some property of the spinach tended to lower the utilization of calcium by the body, the presence of an equivalent amount of crude fiber in the form of filter paper did not act in the same way, or at least not to

an appreciable extent.   The retention of calcium on the control diet was almost twice that on the spinach diets, but only 0.11 mg. per gm. of body weight higher than on the filter paper diet.

The younger rats (Experiment B) retained a greater proportion of the calcium ingested than the older rats (Experiment A), but the relative retentions on the various food mixtures were quite similar to those in the earlier experiment.   Because of the rather surprizing results obtained on Diet 4 in Experiment A, instead of adding this same amount of filter paper to Diet 5, almost 30 times as much was added.   In spite of the abnormally high cellulose content of this ration, the rats receiving it utilized very nearly as much calcium as did those on the low fiber diet.   As for differences between raw and cooked spinach, in the case of the younger rats more calcium was retained when the spinach of the diet was cooked (1.70 mg. per gm. of rat as compared with 1.36 mg. when the spinach was fed raw); for the older rats, the cooked spinach appeared to favor retention no more than the raw.   The percentages of intake retained are shown in Table IV.

The retention of phosphorus did not parallel that of calcium in all cases, either for the older or the younger group of animals.   In both experiments the phosphorus retention per gm. of body weight was highest on the filter paper diet, lowest on the spinach diets, and intermediate on the control diet.   These values are listed in Table IV.   In general, the retention of phosphorus appears to have been poorer on the diets containing spinach than on the low crude fiber diet.   On the other hand, on both filter paper diets, the retention of phosphorus was either approximately the same as, or even greater than on the control diet.

The ratio of calcium to phosphorus retained varied from 0.7:1.0 to 1.5:1.0.

Path of Excretion.—In both experiments the greater part of the calcium excretion of the rats on the spinach diets was through the intestines (from 92 to 96 per cent of the total excreted), and this was true to a somewhat lesser degree on the other diets in Experiment A.   In Experiment B, however, on the control diet only 50 per cent of the total calcium excreted, and on the high filter paper diet 71 per cent, was in the feces.   More of the phosphorus, on the other hand, was excreted by way of the kidneys on the spinach diets during both experiments, but particularly in the first.   The percentages of total excretion in the feces are given in Table IV.

Retention on Low Fiber Versus Spinach Diets.—It has been shown in the present study that the retention of calcium and of phosphorus on the spinach diets was not as good as on the low fiber diet. The differences were more striking in Experiment B than in Experiment A, probably because the animals in Experiment B were younger and had a greater need, therefore, for the storage of minerals. Perhaps herein lies the explanation of certain apparently contradictory results reported in the literature. For the most part, those investigators who found as great a retention of calcium and phosphorus from vegetables as from milk had used adults as subjects, whereas those who did not had performed their experiments on children or young animals. McClugage and Mendel's experiments do not fit into this classification, for although they used grown dogs, they observed less satisfactory calcium balances on a vegetable than on a milk diet. This may, however, be due to the fact that dogs are usually unaccustomed to a high vegetable diet, so that calcium fed in this form might not be as well utilized. In general, though, there was apparently no difference in the availability of the minerals of vegetables and milk when these were needed merely for maintenance. However, when the organism required these elements for the building of new tissue, the vegetables did not seem to be as good a source as milk.

In a chapter dealing with the effect of foods on metabolism, Blunt and Cowan (2) summarize the matter thus:

"From this not large group of experiments, then, it looks as if the calcium and phosphorus of vegetables are satisfactorily absorbed by adult human beings, but not dogs, only fairly well by growing rats, and, especially important, not very satisfactorily by children."

Effect of Adding Crude Fiber.—Since the spinach was the only variable in Diets 1 to 3 of the present study, it is obvious that some property of the spinach was responsible for interfering with the retention of the minerals. Had this factor been the presence of crude fiber in the spinach, one might reasonably have expected a similar lowering of retention on Diet 4, when filter paper was fed in amounts equal to the crude fiber content of the spinach. On this diet, however, the retention of calcium and phosphorus was almost as great as on the control diet. In other words, crude fiber, as

such, did not lessen the availability of the calcium and phosphorus. This does not rule out any effect of the crude fiber of spinach, united as it probably is with the other components of the spinach. Woodruff and Miller (23), in metabolism experiments on rats, found less fiber in the feces after adding potato cellulose to their basal ration than after adding potato. Even feeding huge amounts of crude fiber, as was done in our Diet 5, did not lower either the calcium or phosphorus retention appreciably. This is contrary to the results obtained by Sjollema (21), who found that an increase in the roughage of the diet of an adult rabbit from 3 to 15 per cent (oat straw and sawdust were used) resulted in a greater loss of calcium from the body, although the phosphorus excretion did not follow that of the calcium.

In a recent study by Burton (6) on the effect of rolled oats and refined wheat on the calcium and phosphorus balances of children, the difference in crude fiber content of the two diets may conceivably be a factor responsible for the lower retention of both calcium and phosphorus on the oat diet. Of nine women who served as subjects for a metabolism experiment by Coons (7), the one whose diet contained the most raw vegetables and fruits was found to be storing less minerals and nitrogen than almost any of the others. Coons suggests that "the fiber content of the diet probably meant a much lower available calcium intake than that shown by actual analyses."

Effect of Cooking Spinach.—In our experiment on the younger animals considerably more calcium was retained when the spinach of the diet was cooked, even though analysis showed the percentage of crude fiber to be a little greater in the cooked spinach than in the raw. This was not true of phosphorus, nor, in the experiment on the older rats, was it true of either calcium or phosphorus. However, it is possible that here the additional crude fiber in the cooked vegetable might have overbalanced any slight superiority of cooked spinach over raw as a source of minerals. McClugage and Mendel in their experiments reported no beneficial effect of cooking the vegetables. Although Woodruff and Miller found that both potato and potato fiber were assimilated better when cooked than when raw, this does not mean that the minerals necessarily follow the same procedure. It is suggestive, however, for where there is a greater amount of indigestible material, this is likely to pass

through the gastrointestinal tract more rapidly, carrying a greater proportion of the calcium and phosphorus along with it, and allowing less opportunity for the absorption of these.

Path of Excretion.—On the spinach diets more calcium was excreted by way of the intestines than on the control diet, despite the fact that the pH of the diet remained essentially unchanged. These results are in agreement with those of McLaughlin, who observed a marked difference in the proportion of calcium eliminated through the kidneys—2 to 3 times as high a percentage during the milk as during the spinach period. She accounted for the difference by the preponderance of base-forming elements in the spinach diet, but this is not the whole story, since in the present study, with the same degree of alkalinity of the spinach as of the other diets, there was still a diversion of calcium from the kidneys to the intestines. Phosphorus, on the other hand, was excreted more through the kidneys on the spinach than on the spinach ash diets. This corroborates Boas' observation that spinach diverts phosphorus from feces to urine. On the filter paper diets the percentage of phosphorus in the feces in both experiments and of calcium in the first was approximately the same as for the animals on the low fiber diet. The younger rats on the filter paper diet excreted a greater proportion of their total calcium output through the intestines than those on the control diet, but still a much smaller percentage than the rats on the spinach diet. On the whole, then, crude fiber alone does not appear to change the channel of excretion of these minerals appreciably.

In brief, crude fiber in the form of filter paper did not interfere with the retention of calcium or phosphorus. However, the fiber of the diet did influence the *quantity* of feces excreted by the younger rats, although not by the older ones. As for the path of excretion, more of the calcium was excreted in the feces, more of the phosphorus in the urine, on the spinach than on the spinach ash diets. Cooking the spinach appeared to favor only the retention of calcium, and that only in the younger animals.

SUMMARY AND CONCLUSIONS.

The calcium and phosphorus metabolism of young rats was studied to determine (a) whether the crude fiber in the diet affected in any way the retention of calcium and phosphorus by the

body, and (b) whether the effect of raw and cooked spinach on metabolism was different. The sources of crude fiber used were raw spinach, cooked spinach, and ashless filter paper.

The results of the study show:

1. A very high retention of calcium and phosphorus on the control diet (basal diet with spinach ash, but no crude fiber added).

2. A smaller retention of calcium and phosphorus on the spinach diets.

3. A high retention on the spinach ash and filter paper diets, both when the crude fiber of these diets equalled that of the spinach rations and when it was many times as great.

The low retention from the spinach is not due, therefore, to any unsuitableness in the ash, nor to the cellulose as such, but to some other characteristic of the spinach calcium and phosphorus, such as their state of combination, lowering their availability.

No significant difference between the effect of raw and cooked spinach was noted.

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PLASMA PHOSPHATASE.

I. METHOD OF DETERMINATION. SOME PROPERTIES OF THE ENZYME.

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The phosphatase of the blood, and particularly that of the blood plasma, has been relatively little investigated. Grosser and Husler (1) pioneering with the best technique then available, found that many mammalian tissues would hydrolyze glycerophosphate, but they were unable to demonstrate that blood, either cat or human, possessed any such activity. Forrai (2) found human blood serum was practically inactive in the hydrolysis of glycerophosphate. Martland, Hansman, and Robison (3) found that laked blood had slight phosphatase activity, but their experiments were concerned almost entirely with autolytic phenomena in whole blood, laked and unlaked. They showed that plasma would very slowly hydrolyze a portion of the phosphoric esters of the red cells contained in a protein-free filtrate from whole blood. Demuth (4) found that an enzyme which hydrolyzed hexosediphosphate was present to a minute degree in the serum of adults, and to a slightly larger extent in the serum of infants. This enzyme had, he stated, a broad optimum pH between 6.8 and 7.8. If this finding be correct, it indicates a definite difference between the plasma enzyme and the phosphatase of other mammalian tissues, which has an optimum pH at 8.8 to 9.3.

In preliminary experiments it soon became evident that normal plasma contained a weak glycerophosphatase active over a fairly wide pH range from 7.0 to above 9.0. To compare one plasma with another it was necessary to evolve a reliable quantitative method for the estimation of this enzyme, and this proved at first a matter of some difficulty.

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The determination of the amount of any particular enzyme in a tissue is a proceeding fraught with uncertainty. Apart from questions of enzyme stability, we have to consider possible activators and inhibitors, principal amongst which is usually to be reckoned the pH of the medium. Is the activity of the enzyme to be determined at the optimum pH for its activity or at the apparent pH of the tissue in which it is found—the two pH's very rarely coincide—or at some arbitrary pH?

In working out a standard method for the comparison of the phosphatase activity of two or more samples of plasma, certain theoretical desiderata have been abandoned in favor of convenience. It was decided, for example, after a number of trials, not to endeavour to determine the activity at the optimal pH (see below) of 8.8 to 9.2, but to use separated plasma at its natural pH (of 7.5 to 7.6) plus substrate at about the same pH, to determine the actual pH at which the hydrolysis proceeded, and to calculate, from the amount of free phosphate actually liberated, the amount that would have been produced had the reaction proceeded at exactly pH 7.6, all the pH determinations being made at room temperature. By this means the greatest simplicity and minimal expenditure of plasma compatible with reasonably accurate and comparable results have, I believe, been obtained. It is proposed to give the method in detail, and then to discuss certain points in it.

Method.

(a) *Substrate.*—1.5 gm. of pure crystalline sodium β-glycerophosphate (it is essential to use the β salt) are dissolved in 1 liter of distilled water, and the reaction adjusted with a few drops of 0.2 N HCl to a pH of 7.6 (determined colorimetrically with phenol red). The substrate solution is quite stable if shaken with $\frac{1}{2}$ cc. of chloroform, and preserved in a tightly stoppered bottle in the refrigerator.

(b) *Plasma.*—Enough blood should be drawn to obtain just over 5 cc. of plasma; 11 to 12 cc. are usually sufficient. The blood, drawn, in man, from an arm vein, in other animals in any convenient way which does not entail the passage of blood over damaged tissues, is oxalated in a suitable centrifuge tube, containing 2 drops of 20 per cent potassium oxalate solution (a higher concentration of oxalate is inadvisable) and centrifuged for some

10 minutes at 3000 R.P.M. Hemolysis should be avoided as far as possible; a badly hemolyzed plasma is useless for any comparative work. The carefully separated plasma is filtered slowly through a small plug of cotton wool to remove any leucocytes which may have escaped being carried down during the centrifugation, collected into a small test-tube, and stoppered.

(c) *Determination.*—For each plasma in which the phosphatase content is to be determined, 10 cc. of the substrate are placed in each of five clean test-tubes (two at least of which must be the correct size and shape to fit the pH comparator) of about 15 cc. capacity with well fitting stoppers, preferably glass, and 1 cc. of 1.8 per cent sodium chloride is added. Then exactly 1 cc. of plasma is run into each tube, and mixed. To one only of the tubes, 10 drops of 0.02 per cent phenol red is added, and the pH determined colorimetrically at room temperature, another of the five tubes being used as a blank for the comparator. Half quantities of plasma and substrate may be used, if the amount of plasma is limited, for the pH determination.

To all the four tubes without phenol red, 4 drops of chloroform are now added. To two, 2 cc. of 25 per cent trichloroacetic acid solution are added, and the two tubes shaken, restoppered, and put into the refrigerator. The remaining two tubes are well stoppered, mixed without shaking, and kept in the water thermostat at 38° for 48 hours ± 15 minutes, with occasional mixing. At the end of this time the tubes are removed from the bath, cooled, and 2 cc. of trichloroacetic acid added to each. After standing for 1 hour, the contents of the tubes, and also of those previously placed in the refrigerator ("zero hour tubes") are filtered through 9 cm. Whatman No. 30 papers and the inorganic phosphate determined on 10 cc. of the filtrates by the Briggs or other suitable micro method for phosphate. To bring the amount of inorganic phosphate in the 10 cc. aliquots from the zero hour tubes into the best region for colorimetric P determination it is advisable to add exactly 0.100 mg. of P to each of the two flasks containing the zero hour aliquots before adding the other reagents, and subtract this amount afterwards.

The difference between the inorganic P figures for the aliquots from the zero hour tubes and those from the 48 hour tubes (duplicates should agree within 5 per cent) represents $\frac{10}{14}$ of the hydrolysis

brought about by 1 cc. of plasma.   Having determined this figure, a small correction may be necessary, since the hydrolysis may not have proceeded at the pH of 7.6, which is, by definition, the pH at which our enzymic activity is to be determined.

The correction to be applied is given by the curve shown in Fig. 1 which has been obtained from several experiments in which the activity-pH curve for plasma phosphatase over the range pH 7.3 to pH 8.0 has been determined.

The optimum pH for the plasma enzyme is, like that of the phosphatase of other tissues under the same conditions of substrate
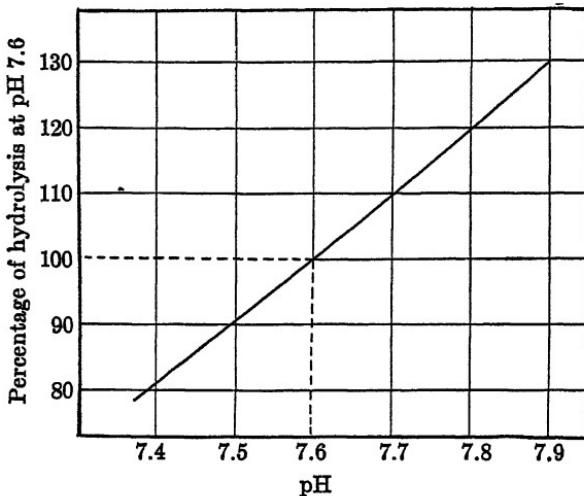
FIG. 1. Curve for making correction to pH 7.6 of the phosphatase value determined at some neighboring pH.   The correction is approximately 10 per cent for each 0.1 pH.

concentration, a fairly broad one at pH 8.8 to 9.2.   Any determinations made as outlined above are made, therefore, at a considerable distance from the theoretically most advantageous region, namely, that of the optimum pH for the particular enzyme substrate system.   They are made at a position on the activity-pH curve where the slope is a fairly steep one, so that it is advisable, in order to obtain any approach to accuracy, that the pH of the reaction mixture be determined, if possible, within 0.05 of a pH unit.

(d) *Plasma Phosphatase Unit.*—A plasma sample containing 1 unit of phosphatase per cc. is one of which 1 cc. will liberate 1 mg.

of inorganic phosphate (expressed as P) from excess sodium β-glycerophosphate solution in 48 hours at 38° and a pH of 7.6, the hydrolysis to be conducted in absence of added buffer, the pH determinations to be made at room temperature, and the other conditions to be those stated above.

This definition differs from that previously given for the quantitative expression of the phosphatase content of more active tissues (5) in that the original unit was some 24 times as great as the present one, and the determination was carried out in the presence of added buffer at pH 8.9 instead of at pH 7.6 without the addition of buffer.

(e) *Remarks.*—The reasons for adopting pH 7.6 for the plasma measurements are as follows: First, the pH of plasma from oxalated blood under the conditions employed is usually, at room temperature, from 7.50 to 7.65. Second, to change this to pH 8.9 is an inconvenient procedure, requiring rather large quantities of plasma and of alkali, and necessitating a correction for dilution. There is, it is true, an increase in the activity of the enzyme at this pH, but this does not outweigh the disadvantages of the alkali titration. Third, in the neighborhood of pH 7.6 both plasma and glycerophosphate are more efficient buffers than at pH 8.9. Fourth, even in presence of added buffer, it is not easy to keep a plasma-glycerophosphate system for 48 hours at pH 8.9, but at pH 7.6 the acidity remains sensibly constant for this period.

The somewhat long period (48 hours) of incubation is advisable since plasma has, relatively to such tissues as bone, intestinal mucosa, or kidney, only relatively slight phosphatase activity—in the normal adult man, kidney tissue is some 300 times as active as the corresponding weight of plasma. The alternatives, namely, the use of larger quantities of plasma or the sacrificing of duplicates, are both unsatisfactory. Moreover, with the quantities of plasma and other materials recommended above, the work can be done in the ordinary size of test-tubes. This, when dealing with several plasma samples at once (each sample requiring at least five test-tubes) is an obvious advantage.

Some Properties of Plasma Phosphatase.

Effect of H Ions.—Under the conditions of concentration of substrate described above, with glycine-NaOH buffers, the opti-

mum pH for the action of the plasma enzyme on glycerophosphate is between pH 8.8 and 9.1.   Experiments illustrating this point are shown in Table I.   This optimum pH is the same for normal human, rat, or rabbit plasma and also for the plasma in cases of bone disease in man.

Effect of Mg Ions.—Like hydrogen ions, Mg ions have a strong stimulating effect on the hydrolysis in low concentrations and an inhibitory effect in higher concentrations.   The stimulating effect

TABLE I.

Effect of H Ions on Activity of Plasma Phosphatase.

	pH determined electrometrically at 38° to nearest 0.1 pH.		Hydrolysis in mg. P per cc. plasma.
	At start.	After 48 hrs.	
Normal human plasma.	6.8	6.9	0.090
	8.0	8.0	0.179
	8.5	8.5	0.234
	8.9	8.85	0.269⎫
	9.1	9.0	0.271⎰
	9.6	9.5	0.213
	9.85	9.80	0.098
Plasma from case of osteitis deformans.	8.2	8.2	2.50
	8.6 ·	8.7	2.81⎫
	8.9	8.9	2.79⎰
	9.3	9.3	2.35 '
	9.7	9.6	1.53
	10.0	9.8	0.80

of Mg ions for the active glycerophosphatase of kidney tissue has already been shown by Erdtman (6).

The two experiments given in Table II demonstrate this effect with rabbit plasma and with rat serum.   The Mg was added as $MgCl_2$, the experiment conducted at pH 7.6, 1 cc. of rabbit plasma and $\frac{1}{2}$ cc. of rat serum being used, for 48 hours at 37.5°.

This effect of Mg ions on plasma phosphatase was not discovered until most of the determinations on plasma phosphatase in disease, to be recorded in another paper, had been made.   It is, however, in the highest degree unlikely that increases in the apparent phosphatase content of certain pathological plasma samples are

explicable on the basis of an increase, not of phosphatase, but of Mg ions in the blood. Thus in the first experiment in Table II an increase of "apparent phosphatase" of 27 per cent was brought about by a rise in Mg ion concentration of some 3000 per cent. In the second experiment in Table II the maximum rise of apparent phosphatase of 180 per cent required an Mg ion increase of some 60,000 per cent. Such changes certainly do not occur in blood. Reiche (7) reports that whilst the variations amongst twenty healthy individuals were from 2.0 mg. to 2.9 mg., with a mean of 2.45 mg., the magnesium content of the serum varied, in 400 cases of disease, between 1.8 and 3.1 mg. only per 100 cc. 2.45 mg.

TABLE II.

Effect of Mg Ions on Plasma or Serum Phosphatase.

Rabbit plasma.		Rat serum.	
Concentration of Mg ions in reaction mixture.	Hydrolysis for 1 cc. of plasma (mg. P).	Concentration of Mg ions in reaction mixture.	Hydrolysis for ½ cc. rat serum (mg. P).
M		M	
0.0001 (approximate).	0.137	0.00005 (approximate).	0.189
0.00033	0.144	0.00033	0.256
0.0010	0.154	0.0010	0.356
0.0033	0.174	0.0033	0.402
0.01	0.174	0.01	0.485
0.033	0.162	0.030	0.535
		0.090	0.288

being taken as the pathological mean, the range indicated is within ± 30 per cent of this figure. Changes in the Mg content of the plasma of this magnitude would be hardly appreciable as far as their effects on plasma phosphatase are concerned.

Effect of Ca Ions.—The effect of Ca ions on the glycerophosphatase activity of serum is slightly inhibitory, in all concentrations from 0.0002 M to 0.12 M in the reaction mixture at pH 7.6. The inhibition increases with the concentration of calcium. (Table III). The calcium was added as $CaCl_2$ (at pH 7.6), the experiment conducted at pH 7.6, with 1 cc. of ox serum, for 48 hours at 37.5°.

It is clear from Table III that even fairly large variations in the

serum calcium in disease would have little effect on the apparent value of the plasma phosphatase.

TABLE III.

Effect of Ca Ions on Plasma Phosphatase.

Final concentration of Ca ions in reaction mixture.	Hydrolysis for 1 cc. plasma in mg. P.
M	
0.0002 (approximate).	0.192
0.0004	0.188
0.0012	0.176
0.004	0.181
0.012	0.163
0.04	0.154
0.12	0.133

TABLE IV.

Rate of Hydrolysis of Different Phosphoric Esters by Plasma Phosphatase.

Ester.	How obtained or prepared.
1. Sodium hexosediphosphate.	(a) Fermentation in laboratory. (b) Bayer products.
2. " α-glycerophosphate. ⎫ 3.* " β-glycerophosphate. ⎬ 4.* " pyrophosphate. ⎭	Pure calcium α salt and sodium β salt.† Prepared in laboratory from pure $Na_2HPO_4 \cdot 12H_2O$.
5. " glycophosphate.	Prepared in laboratory by synthetic action of intestinal phosphatase on a mixture of pure glycol and Na_2HPO_4 solution.
6. " salt of guanine nucleotide.	Prepared in laboratory from yeast nucleic acid.

  * Esters 3 and 4 were hydrolyzed at almost the same rate.
  † These salts were obtained from Boots Pure Drug Company through the kindness of Dr. F. L. Pyman.

Effect of Na, K, and Cl Ions.—Small variations in the concentration of Na, K, or Cl ions have no appreciable effect on the phosphatase activity of the plasma.

Action on Different Substrates.—The phosphatase of plasma, like that of kidney, intestinal mucosa, and bone, will hydrolyze a large number of mono-esters of phosphoric acid, including in this category, pyrophosphates. In all, six phosphoric esters have been hydrolyzed, the experiments having been arranged so that the amount of free phosphate liberated by the plasma enzyme from the phosphoric ester in question has been compared with that liberated by the same enzyme from sodium β-glycerophosphate under exactly the same conditions of time, pH, temperature, and original concentration of esterified P in the reaction mixture. These esters are arranged in Table IV in order of rapidity of

TABLE V.

Effect of Mg Ions on Hydrolysis of Three Different Substrates by Plasma Phosphatase.

Temperature 38°; ester P concentration in reaction mixture = 0.148 mg. P per cc; pH 7.6. Rat plasma used.

Substrate.	Concentration of Mg.	Amount of hydrolysis per 10 cc. reaction mixture (duplicates).	
	M	*mg. P.*	
Na α-glycerophosphate.	About 0.00004	0.520	0.540
	" 0.003	0.845	0.840
" β-glycerophosphate.	" 0.00004	0.254	0.250
	" 0.003	0.667	0.667
" pyrophosphate.	" 0.00004	0.269	0.263
	" 0.003	0.348	0.354

breakdown, the most actively attacked being at the head of the list.

In all the above cases, excluding that of hexosediphosphate, the rate of hydrolysis was definitely stimulated by the addition of Mg ions. In different substrates, however, the percentage effect was not the same. In the case of hexosediphosphate the stimulation due to Mg ions, if any, was very small. This matter is being further investigated.

Details of a typical experiment are given in Table V.

The Mg ion stimulus is clearly evident for each of these three substrates, although the magnitude of the effect is not the same in each case.

Effect of Age.

In young normal children, and in young animals generally, the blood plasma has a higher phosphatase content than in the adult.

Man.—For sixteen normal adult men and women, ages 20 to 61, the values found ranged from 0.10 to 0.21, with an average value of 0.15.   In eight children, normal or nearly so, (certainly not suffering from bone disease) the values ranged from 0.17 to 0.34, with an average value of 0.26.

Rabbit.—A group of twelve young rabbits, aged between 4 and 6 weeks had values varying from 0.14 to 0.50, with an average of 0.31, whilst twelve adults of the same breed had values ranging from 0.06 to 0.14, with an average of 0.11.

TABLE VI.
Effect of Age on Plasma Phosphatase.

Series A.		Series B.	
Age.	Plasma phosphatase.	Age.	Plasma phosphatase.
wks.	*units*	*wks.*	*units*
4	1.14	5	0.75
6	0.93	6	0.64
8	0.69	8	0.60
10	0.72	9	0.50

Rat.—Perhaps the clearest instance of the effect of age is shown by the figures in Table VI, derived in each case from the mixed plasma samples of three or four rats.   The litters were distributed throughout the groups.   The animals were fed on the normal stock diet (Bills' (8) modification of Steenbock's stock diet).

Once the adult state is reached, the plasma phosphatase does not appear to vary very much from one day to another.   My own value, determined at six various times between January, 1929 and June, 1930, has ranged from 0.11 to 0.16, with an average of 0.13. A normal adult rabbit, bled seven times at approximately fortnightly intervals between January and April, 1930, has given plasma phosphatase values varying from 0.06 to 0.13, with an average of 0.09.

Such figures could be multiplied, but those given suffice to illustrate the points that the phosphatase value is usually higher

in the plasma of young animals than in adults, and that in adults the value for any individual remains fairly constant, at least over several months of time.

Plasma Phosphatase in Different Animals.

No systematic attempt has been made to compare different animal species with regard to the phosphatase content of the

TABLE VII.

Plasma Phosphatase in Different Animals.

	No. of individuals.	Range.	Average values.
		units	*units*
Man, normal adult..............	16	0.10–0.21	0.15
Rat, " "	6	0.25–0.67	0.40
Dog, " "	4	0.11–0.16	0.14
Rabbit, " "	20	0.06–0.16	0.11

TABLE VIII.

Effect of Leucocytes and of Red Blood Cells.

Hydrolytic agent.	Phosphatase units per cc.
	units
Filtered plasma...	0.09
" " + 0.003 M MgCl₂......................	0.17
Plasma containing leucocytes.........................	0.23
Red blood cells.......................................	0.55
" " " + 0.003 M MgCl₂.......................	1.10

plasma. The figures in Table VII indicate, however, that marked differences exist.

Rat plasma is usually 2 to 3 times as active as human plasma; rabbit plasma is usually distinctly less active. The phosphatase value for the plasma of normal young rats approaches that found for milder cases of osteitis deformans in man.

Effect of Leucocytes and of Hemolysis.

In the method just outlined for the determination of phosphatase in plasma, it has been mentioned that every care should be

taken to avoid both hemolysis and the presence of leucocytes in the plasma. That these precautions are not without reason is illustrated by the following experiment.

40 cc. of fresh·rabbit blood (oxalated) were centrifuged, the plasma removed, and the leucocyte layer freed as far as possible from red blood cells by a second centrifugation in a small tube. The leucocytes from 40 cc. of whole blood were thus obtained almost completely free from red blood cells, and were taken up in 4 cc. of plasma. The separated red blood cells were laked by adding an equal volume of water and some chloroform, and 2 cc. of the laked mixture, equivalent to 1 cc. of red blood cells, were used as the enzyme preparation. Results are shown in Table VIII.

The plasma phosphatase is more than doubled by the addition of leucocytes to the reaction mixture, whilst the red blood cells may be 6 or more times more active than the plasma of the same blood.·

SUMMARY.

Details are given of a simple method for the determination of the phosphatase content of the blood plasma of man and of other animals. In normal plasma the amount of the enzyme per unit quantity of plasma is small compared with the amount present in an equal weight of tissues such as kidney, intestine, or bone. In determining the phosphatase content of plasma it is necessary to avoid both hemolysis and the presence of leucocytes in the plasma, since both red and white cells contain more phosphatase than plasma.

Certain properties of the plasma enzyme are described. It appears to have the same optimum pH for activity as that of the phosphatases of other mammalian tissues (pH 8.8 to 9.2). Mg ions act as a powerful stimulant to its activity, with an optimal pMg between 1 and 2. Calcium ions act as a mild inhibitor. The enzyme will hydrolyze all the phosphoric esters which have so far been presented to it, namely hexosediphosphate, synthetic α- and β-glycerophosphates, pyrophosphate, glycophosphate, and guanine nucleotide. The hydrolysis of each of these substrates with the possible exception of hexosediphosphate is stimulated by Mg ions.

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PLASMA PHOSPHATASE.

II. THE ENZYME IN DISEASE, PARTICULARLY IN BONE DISEASE.

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In a previous paper it has been stated that the activity of the plasma in hydrolyzing phosphoric esters is, compared with the activity of such tissues as the bone, the intestinal mucosa, and the kidney, relatively weak. In diseases of phosphatase-rich tissues, particularly those conditions involving a progressive and widespread breakdown of such tissues, with possible leakage of intracellular materials into the blood, it would not, perhaps, be surprizing if an increase above normal occurred in the relatively low phosphatase content of the plasma. On the other hand, a fall in the phosphatase of the plasma below normal would mean a diminution in an already small activity, and would probably be of rarer occurrence.

The three most active, and at the same time quantitatively important tissues, as far as their phosphatase content is concerned, are undoubtedly the intestinal mucosa, the kidney, and the epiphyseal zone and periosteum of bone. The liver and the central nervous system have a relatively low phosphatase activity, and the muscles are still less active (1). In considering possible sources from which this enzyme might leak, under pathological conditions, into the blood, it is unlikely that there are any tissues other than the three first mentioned which have any quantitative significance.

A preliminary account of the changes which occur in the phosphatase of the plasma in certain diseased conditions has already been published (2). I found that in seven cases of osteitis defor-

mans and in one case of generalized osteitis fibrosa the plasma phosphatase was raised well above the normal figure, the values

TABLE I.

Enzyme in Plasma in Normal Cases.

Age.	Sex.	Description.	Date.	Enzyme content of plasma.	Remarks.
yrs.				units	
35	M.	Normal.	Jan., 1929	0.16	
35	"	"	Mar., "	0.13	
35	"	"	June, "	0.14	Same individual.
36	"	"	Sept., "	0.11	
36	"	"	Nov., "	0.13	
36	"	"	June, 1930	0.11	
24	"	"		0.13	Nystagmus.
20	F.	"		0.15	Cured menor-rhagia.
30	M.	"		0.21	
38	F.	"		0.15	Slight hysteria.
27	"	"		0.16	
41	M.	"		0.11	
40	"	"		0.15	
26	"	"		0.18	
45	"	Chronic bronchitis.		0.19	
61	"	Normal.		0.18	Some evidence of rickets in child-hood.
22	"	"		0.15	
29	F.	"		0.10	
24	"	Neurotic.		0.16	
38	"	Normal.		0.12	
27	M.	"		0.11	
		Lowest normal.		0.10	
		Highest "		0.21	
Arithmetical average of all normals..............				0.150	

found being from 4 to 20 or more times the normal. In the present paper further work is described, confirming the preliminary findings, and extending them to other types of disease. The nu-

merical results are first tabulated, and then discussed. As the total of the cases investigated now numbers over 400, no attempt will be made to record each individual case, and except in the most interesting groups, typical examples only of the findings in various pathological conditions will be given. The method of determination used is that outlined in Paper I, the enzyme content of the plasma being expressed in phosphatase units as already defined (3).

TABLE II.
Enzyme in Plasma in Diabetic Cases.

Age.	Sex.	Description.	Enzyme content of plasma.	Remarks.
yrs.			units	
29	F.	Diabetes mellitus.	0.20	On insulin.
67	"	" "	0.15	" "
65	M.	" "	0.20	" "
30	"	" " (severe).	0.28	" " Plasma quite opaque.
48	F.	Diabetes mellitus.	0.23	On insulin.
37	M.	" "	0.23	" "
45	F.	" "	0.17	" " Carcinoma of breast.
25	M.	" . "	0.31	On insulin. Plasma quite opaque; cholesterol tophi in various parts of body.
14	F.	" "	0.24	On insulin.
40 (?)	"	" "	0.16	" " Very anemic.
48	"	" "	0.37	On insulin.
48	"	" " (2 months later).	0.51	" " Same individual.

The results of phosphatase determinations made on plasma samples from seventeen normal individuals are shown in Table I. It will be noted that the maximum variation lies between ± 40 per cent of the mean value of 0.15.

Thirty-eight other cases, similar to those shown in Table II, were observed, varying from 0.11 to 0.55, with no outstanding characteristics except for the case showing the highest figure of

0.55, which was a severe case with hemochromatosis.   The maximum figure for diabetes mellitus of all types of severity was thus 0.55, the minimum 0.11.   The arithmetical average figure is 0.26, but a distribution curve shows a very definite peak between 0.17 and 0.21.   In uncomplicated diabetes the plasma phosphatase may be said, therefore, to be only slightly above normal.

Twenty-five other cases of renal disease besides those reported on in Table III were observed, with a highest figure of 0.33 and a lowest figure of 0.12.   The arithmetical average for this group of thirty-two cases of renal disease (excluding "renal" rickets and renal neoplasms) is 0.21; a distribution curve shows a peak between 0.18 and 0.21.

TABLE III.

Enzyme in Plasma in Cases with Renal Diseases (Excluding "Renal" Rickets and Renal Neoplasms).

Age.	Sex.	Description.	Enzyme content of plasma.
yrs.			*units*
29	F.	Chronic nephritis.	0.16
53	M.	Renal calculus.	0.26
24	F.	Acute nephritis, purpura.	0.33
21	"	Chronic "	0.24
40	"	Renal calculus, pyonephrosis.	0.18
25	"	Albuminuria of pregnancy.	0.27
29	M.	Acute nephritis.	0.29

Ten other cases of arthritis besides those reported in Table IV, ranging from 0.09 to 0.19, were observed, giving an average for the twenty-five cases of various types of arthritis of 0.16—a normal figure.

Although the arithmetical average is normal, the distribution curve shows a rather greater number of cases in the lower groups (0.09 to 0.13) than in the normal cases shown in Table I.

Fifteen other cases of neoplasms besides those reported in Table V were studied, varying from 0.59 (papilloma of bladder) to 0.13, with an average for 22 cases of 0.28,   This average figure is distinctly above the normal.   Neoplasms of the kidney and bladder show the highest figures in this group, namely, papilloma of blad-

TABLE IV.

Enzyme in Plasma in Cases with Arthritis.

Age.	Sex.	Description.	Enzyme content of plasma.	Remarks.
yrs.			units	
51	F.	Rheumatoid arthritis.	0.16	
44	"	Arthritis deformans.	0.11	
58	"	Rheumatoid and osteoarthritis.	0.18	
53	"	" arthritis.	0.33	Without any definite bony change.
29	M.	" "	0.12	" "
46	"	" "	0.16	" "
26	F.	Acute infective arthritis.	0.11	
26	M.	Chronic rheumatoid arthritis.	0.15	
50	"	" osteoarthritis.	0.22	
45	F.	" "	0.12	
43	"	" "	0.09	
48	M.	" "	0.25	
58	F.	Osteo- and rheumatoid arthritis.	0.18	
73	"	Osteoarthritis.	0.18	Bony thickening of knees.
71	"	Chronic osteoarthritis.	0.19	

TABLE V.

Enzyme in Plasma in Cases with Neoplasms (Excluding Bone Neoplasms).

Age.	Sex.	Description.	Enzyme content of plasma.
yrs.			units
65	F.	Carcinoma of ovary.	0.31
60	M.	Thoracic neoplasm.	0.21
61	"	Carcinoma of stomach.	0.15
39	F.	Renal tumor.	0.36
53	"	Carcinoma of breast.	0.22
7	"	Hypernephroma.	0.41
49	M.	Mediastinal neoplasm.	0.26

TABLE VI.

Enzyme in Plasma in Cases with Diseases of Thyroid Gland.

Age.	Sex.	Description.	Enzyme content of plasma.	Remarks.
yrs.			*units*	
42	F.	Exophthalmic goiter, severe.	0.75	
20	M.	" " "	0.32	
31	"	" " "	0.46	
47	"	" " "	0.70	Bone rarefaction.
36	F.	" " "	0.27	
21	"	" " "	0.43	6 days after partial thyroidectomy.
20	M.	Hyperthyroidism.	0.32	
20	F.	"	0.18	Iodine treatment. Basal metabolism +30 per cent.
22	"	"	0.27	Basal metabolis +56 per cent.
38	M.	Adult cretin.	0.16	
29	"	Exophthalmic goiter.	0.40	Basal metabolism +78 per cent.

TABLE VII.

Enzyme in Plasma in Cases with Osteomyelitis.

Age.	Sex.	Description.	Enzyme content of plasma.
yrs.			*units*
11	M.	Acute osteomyelitis.	0.34
20	"	Chronic "	0.14
13	"	" " with sequestration (tibia).	0.41
13	"	Chronic osteomyelitis of femur. Same 2 mos. later.	0.26 / 0.28
15	"	Chronic osteomyelitis of femur and tibia.	0.18
12	F.	Chronic osteomyelitis.	0.25
9	M.	" "	0.32

der, 0.59, renal carcinoma, 0.57, hypernephroma, 0.41, renal tumor, 0.36. Excluding these four cases the arithmetical average of the remainder sinks to 0.21, only slightly above the normal average.

TABLE VIII.

Enzyme in Plasma in Fragilitas Osseum.

Case No.	Age.	Sex.	Enzyme content of plasma.	Remarks.
	yrs.		units	
1	9	M.	0.27	
1	9	"	0.33	Same case 1 mo. later.
2	7	"	0.54	
3	6	F.	0.66	
4	6	"	0.51	
5	27	M.	0.16	

TABLE IX.

Enzyme in Plasma in Rickets.

Case No.	Age.	Sex.	Enzyme content of plasma.	Remarks.
	yrs.		units	
1	1.7	M.	0.77	
2	0.4	F.	1.7	•
3	0.4	"	0.88	
4	0.3	M.	0.49	.
5	0.5	F.	1.4	
6	0.4	M.	0.75	
7	0.2	"	0.42	Craniotabes.
8	0.5	"	1.5	Very severe craniotabes.
9	0.6	"	0.97	Tetany.
10	0.6	"	0.62	"

The average for the ten cases of hyperthyroidism reported in Table VI is 0.41, a figure considerably above the normal.

In Tables VII to XIV, cases of bone disease or diseases in which the bones are known to be directly involved, will be given. In this connection, with regard to Table VI, there is known to be, in many cases of hyperthyroidism, a negative calcium balance (4) with diminution in the density of the bone.

'There is no marked rise in osteomyelitis, as may be seen from Table VII, though some of the figures are slightly above normal for the age.   The average figure is 0.27; normal average for this age is about 0.17.

There is a tendency to a raised value in fragilitas osseum as shown in Table VIII, but no constant increase.   It will be observed that the highest values are given with the youngest patients.   The average for the four children is 0.50; the normal value for this age is in the neighborhood of 0.22.

Case 8, Table IX, was a very typical case of severe rickets. Cases 9 and 10, Table IX, were of the low calcium type.   The average for the ten cases of rickets is 0.95.   The normal for the same age of infant is 0.26, the variations being from 0.17 to 0.34.

Enzyme in Plasma in Renal Rickets.

Case No.	Age.	Sex.	Enzyme content of plasma.
	yrs.		*units*
11	17	M.	1.5
12	11	F.	0.9

Both the above cases, considered clinically to be typical cases of renal rickets, show a definite large increase in the plasma phosphatase.

Enzyme in Plasma in Adolescent Rickets.

Age.	Sex.	Enzyme content of plasma.	Remarks.
yrs.		*units*	
14	M.	>2.4	Recrudescence of infantile condition. Kidneys apparently normal. x-Ray shows typical rickets.

Case 1, Table X, is the case which has recently been reported in detail by Hunter (5).

From the data in Table XI there appears to be no evidence of a fall below the normal plasma value in postoperative parathyroid tetany.

TABLE X.

Enzyme in Plasma in Generalized Osteitis Fibrosa.

Case No.	Age.	Sex.	Date.	Enzyme content of plasma.	Remarks.
	yrs.		*1929*	*units*	
1	40	F.	Mar. 4	>1.3	Limit of method. Hydrolysis of substrate complete.
			May 15	>1.3	" "
			" 28	>1.3	" "
			June 5	1.9	Modified method.
			" 15	>2.5	Limit of modified method.
			1930		
			Feb. 21	1.1*	Operation Nov. 29, 1929. Hypertrophied parathyroid gland removed.
2	31	M.	June 5	1.1	
			" 18	1.5	

* I am indebted to Dr. S. Levy Simpson for this figure.

TABLE XI.

Enzyme in Plasma in Hypoparathyroid Conditions.

Age.	Sex.	Description.	Enzyme content of plasma.	Remarks.
			units	
42 yrs.	F.	Hypoparathyroidism and hypothyroidism following thyroidectomy.	0.21	Severe case.
		Same, 3 wks. later.	0.34	–
34 "	"	Hypothyroidism and hypoparathyroidism following thyroidectomy. Same, 3 wks. later.	0.14	" "
			0.13	
Adult.	M.	Postoperative parathyroid tetany.	0.19	
"	F.	" " "	0.17	
"	M.	" " "	0.19	
"	F.	" " "	0.14	

Case 1, Table XII, is a doubtful case of osteomalacia.   There has been progressive rarefaction of the bones for some years. x-Ray plates recall generalized osteitis fibrosa.   There is evidence of severe rickets in childhood.   There is no increased urinary excretion of calcium.   The case, apparently, is slowly yielding to dietetic treatment.

Case 2, Table XII, was originally thought to be a case of osteitis fibrosa, but on a low Ca diet there was no large urinary excretion of Ca.   On a high Ca diet with viosterol there were definite clinical signs of improvement, as well as a marked diminution in the plasma

TABLE XII.

Enzyme in Plasma in Osteomalacia.

Case No.	Age.	Sex.	Date.	Enzyme content of plasma.	Remarks.
	yrs.		*1929*	*units*	
1	12	F.	Nov. 20	1.8	On diet rich in Ca and P and vita-
			1930		mins. Clinical signs of im-
			Jan. 6	1.65	provement.
			" 24	1.29	
2	21	F.	" 9	1.2	
			" 23	1.0	
			Mar. 6	1.1	
			April 17	0.51	On high Ca diet and viosterol since March 20.
			July 24	0.31*	" "

* Figure added at proof correction.

phosphatase.   I am indebted to Dr. R. F. Farquharson for the opinion that, in recent x-ray photographs, the bones of this patient are beginning to show signs of increased calcification.

Case 21, Table XIII, was first rather hastily diagnosed as severe chronic infective arthritis, and a blood sample was taken from this patient as an example of this disease.   After the very high plasma phosphatase finding, a more careful examination revealed quite definite Paget's disease, confirmed by x-ray photographs.

Another case not recorded here, a patient in an infirmary, was diagnosed, from an x-ray photograph of the femur, as suffering from osteitis deformans.   His plasma phosphatase was 0.28.   He died a few days later and on postmortem examination no signs of

osteitis were discovered, but a tuberculous lesion of the head of the femur.

TABLE XIII.

Enzyme in Plasma in Osteitis Deformans.

Case.	Age.	Sex.	Description.	Enzyme content of plasma.	Remarks.
	yrs.			units	
1	52	M.	Osteitis deformans.	>1.3	Limits of method reached, hydrolysis of substrate complete.
2	49	"	" "	0.71	
3	41	"	" "	>1.3	Hydrolysis of substrate complete.
4	59	F.	" "	1.9	
5	50	M.	" "	1.22	
6	55	"	" " and enlargement of prostate	0.70	
7	49	F.	Osteitis deformans.	>1.3	Limits of method.
8		M.	" "	1.13	
9	55	F.	" "	1.18	
10	51	M.	" "	>2.6	" " modified method.
11	58	"	" "	0.84	
12		F.	" "	>1.3	Limits of method.
13	62	M.	" "	0.87	
14	53	"	" " and broken femur.	>1.3	" " "
15	44	F.	Osteitis deformans.	>2.7	" " modified method.
16		"	" "	>3.2	" "
17	63	M.	" "	1.50	
18	50	"	" "	0.65	
19	53	F.	" "	>3.1	Probably the most severe case in this series.
20	59	M.	" " and recent fractures.	1.7	
21	49	F.	Osteitis deformans.	>1.3	Limits of method.

Dr. S. Levy Simpson has informed me that in a series of eleven further cases of osteitis deformans, using practically the same method as that described in Paper I, he has obtained the following figures; 2.3, 1.1, 1.0, 0.43, >2.5, >2.5, 0.51, 0.32, 0.95, 1.8, 2.45.

It is quite clear that the osteitis deformans, generalized osteitis fibrosa, osteomalacia, and rickets cases have a plasma phosphatase

TABLE XIV.

Enzyme in Plasma in Other Lesions of Bone.

Case No.	Age.	Sex.	Description.	Enzyme content of plasma.	Remarks.
	yrs.			units	
1	49	F.	Advanced secondary carcinomatosis of spine and pelvis.	>1.3	Limits of method.
2	41	"	Secondary carcinoma of spine.	0.44	
3	56	"	" " " lumbar vertebræ and pelvis.	0.74	
4	50	"	Secondary carcinoma of pelvic bones.	0.25	
5	16	M.	Periosteal sarcoma of femur.	0.24	
6	17	F.	Sarcoma of femur.	0.63	
7	16	"	Chondroma from upper end of tibia.	0.15	
8	30	"	Hodgkin's disease, with involvement of 3rd and 4th cervical vertebræ.	0.23	
9	25	M.	Hodgkin's disease, with complete destruction of 12th thoracic vertebra.	0.23	
10	48	"	Destruction of 5th cervical vertebra. Cause unknown.	0.13	
11	63	"	Fractured neck of femur.	0.17	
12		"	" " " "	0.14	
13	60	"	Potts fracture, 3 mos. ago.	0.23	Callus formation ++.
14	63	"	Fracture of tibia and clavicle, diabetes.	0.20	
15	58	F.	Tabes, Charcot knees.	0.13	
16	60	F.	Acromegaly and glycosuria.	0.22	
17	38	"	"	0.32	
18	21	M.	Hyperpituitary gigantism.	0.38	

content of a quite different order from that of the cases reported in Tables I to VIII.

In Table XV are given a few miscellaneous cases, none of whom was known to be suffering from bone disease, or a disease directly involving the bone.

Over 100 other miscellaneous cases besides those reported in Table XV showed a plasma phosphatase content ranging from 0.08 to 0.55 units.

Summarizing the results shown in the foregoing tables, Tables I–XV, we find that in only one case (excluding hyperthyroid conditions) where there were not definite bone lesions, was the phosphatase of the plasma above 0.59. This case (Case 6 in Table XV)

TABLE XV.

Enzyme in Plasma in Miscellaneous Cases of Disease, Not Embraced in Any of the Foregoing Categories.

Case No.	Age.	Sex.	Description.	Enzyme content of plasma.	Remarks.
	yrs.			*units*	
1	60	M.	Portal cirrhosis.	0.31	
2	55	F.	Hyperpiesis.	0.18	
3	46	"	Splenomegaly.	0.27	
4	61	M.	Enlarged prostate.	0.16	
5	24	F.	Repeated miscarriage.	0.28	
6	33	"	Hypercholesterolemic splenomegaly.	1.1	Repeated, gave 1.2 units.
7	51	M.	Pneumonia.	0.27	
8	46	"	Acholuric jaundice.	0.10	
9	13	"	Congenital hydrocephalus.	0.32	
10	27	F.	9th mo. of pregnancy.	0.18	
11	36	"	8th " " "	0.25	
12	31	"	7th " " "	0.21	
13	27	"	6th " " "	0.11	
14	19	"	Tachycardia.	0.14	
15	32	M.	Duodenal ulcer.	0.12	

with a plasma phosphatase of 1.2 was an extraordinary case of hypercholesterolemia with splenomegaly, and cholesterol xanthomata; a description of this case has recently been published (6).

DISCUSSION.

Active physiological agents of the type of vitamins and hormones have been much investigated of recent years, and in many cases their rôle in the organism has been well established and defined.

So far, however, though much time has been devoted to work on enzymes, there is, with one possible exception, no clear case of the function of any single intracellular enzyme being even approximately defined. The enzymes liberated by the digestive glands, and thus no longer under direct control by the cell, have obvious functions in the hydrolysis of foodstuffs, but those enclosed within the cell—unless we look upon them as being present adventitiously and purposelessly, a *jeu d'esprit* of nature—have no less important functions to perform.

The activities of the so called proteolytic intracellular enzymes, for example, must be very intimately linked with the life of the cell, presiding as they most probably do, over an equilibrium between the amino acids reaching the cell, and the constitutional or structural cell proteins. Phosphatase may, in the majority of the tissues, be regarded also as an intracellular enzyme and probably in most of the cells in which it occurs it presides over a similar equilibrium between inorganic phosphate and the constitutional phosphoric esters of the cell, a category which includes nucleic acid and the phospholipids no less than the simpler "acid-soluble" phosphoric esters. But the phosphatase in one tissue at least seems to subserve a more specific function.

Since 1923, when Robison (7) discovered that bone contained an active phosphatase and suggested its importance in bone formation, a good deal of evidence, mainly circumstantial but in its total volume very compelling, has slowly accumulated in support of this suggestion. There seems to be little doubt that in considering problems of bone formation, maintenance, and pathology, the very active phosphatase in the epiphyseal zone and periosteum can no longer be ignored but must be accepted as playing a very important rôle in these processes.

The evidence may be briefly summarized as follows: (a) An enzyme is present in bone extracts which will hydrolyze the soluble calcium salts of phosphoric esters to give a calcium phosphate insoluble on the alkaline side of neutrality (7) (whether $Ca_3(PO_4)_2$, $CaHPO_4$, or some other insoluble phosphate of calcium does not concern us at present). (b) This enzyme is present in growing bone in those localities in which deposition of calcium phosphate is proceeding most rapidly (8). (c) A rachitic bone split lengthways and immersed for a short time in a solution of calcium glycerophosphate or calcium hexosemonophosphate will show deposi-

tion of calcium phosphate in the zones of preparatory calcification and of hypertrophic cartilage cells, and in the periosteum (8). (*d*) A substrate for the action of this enzyme is present in both plasma and red blood cells (9, 10). (*e*) The enzyme is present in greatest amount in bone at the time when deposition of calcium salts is most active (10). (*f*) In cartilage which does not calcify, *e.g.* tracheal cartilage, the enzyme is *not* present (7). (*g*) In cartilage which does eventually ossify the enzyme is found simultaneously with the earliest appearance of calcifying centers in the cartilage (11). (*h*) The enzyme is produced by the embryo bone growing *in vitro* in tissue culture media (12). (*i*) Lead, strontium, lithium, silver, and radium administered experimentally or accidentally to the animal tend to be deposited preferentially in the bones (13–17). Each forms a phosphate insoluble in faintly alkaline solutions. As a result of the physicochemical process just outlined their presence in bone is readily explained, since any base circulating in the blood which forms an insoluble phosphate would tend to be precipitated and maintained in this insoluble form where there is a permanent local increase in phosphate ions brought about in a region where diffusion is difficult, *i.e.* in the bones. (*j*) Finally there is the finding described in the present paper that in certain generalized diseases of bone, and, almost without exception, in no other disease, the plasma phosphatase—an enzyme having all the properties of the bone enzyme and apparently identical with it—is markedly raised. At the lowest estimate of its significance, this last finding establishes yet another strong correlation between bone maintenance and phosphatase. It also tends to reinforce the opinion which has been growing of late, that to understand the biochemistry of bone formation, maintenance, and pathology emphasis should be laid as much on the metabolism of phosphoric acid as on that of calcium.

In view of the suggested rôle of bone phosphatase, is the presence of a greatly increased quantity of phosphatase in the plasma in bone disease a cause or an effect of the disease? It might be established as a cause if it could be shown that, owing to its increased activity in bone disease, the plasma enzyme actually hydrolyzed all or part of the circulating substrate for the bone enzyme before the substrate arrived in the vicinity of the bone. A few determinations have been attempted on this point, and it has been found in three cases with a high plasma phosphatase that

there appeared to be as much organic acid-soluble phosphorus in the plasma in these individuals as in normal plasma—namely 0.3 mg. of P per 100 cc. The plasma substrate is in all cases present in such small quantities that the figures found are uncomfortably near the experimental error of the method (0.10 to 0.15 mg. of P per 100 cc.) (see Table XVI).

· That the high plasma phosphatase should be a cause of bone disease is rendered still less likely by the fact that in widespread malignant disease of the bone (*e.g.*, Cases 1, 2, 3, and 6 in Table XIV) where the bone lesion is of known origin the plasma phosphatase is high. In these cases the rise in plasma phosphatase is almost certainly a secondary phenomenon.

<div align="center">

TABLE XVI.

"Ester" Phosphorus in Plasma in Cases of Bone Disease.

</div>

Description of case.	Plasma phosphatase.	Mg. P per 100 cc. plasma.		
		Inorganic P.	Total acid-soluble P.	"Ester" P.
	units			
Osteitis deformans................	2.6	3.70	3.95	0.25
Generalized osteitis fibrosa.........	2.5	3.10	3.50	0.40
Atypical osteitis fibrosa...........	1.09	2.70	2.90	0.20
Mean of 17 normal cases...........	0.15			0.33

If not a cause, then it may be a direct or indirect effect of the bone disease. In some way, in short, the enzyme may leak out of the bone into the plasma, or leak out at more than the normal rate, possibly because it is produced in excessive amounts in the bones in attempted compensation for the lesion, or possibly because owing to weakness of the bones there is a greater amount of bending and crushing which mechanically squeezes out some of the cell contents. The continual leakage of enzyme from the bone in osteitis deformans may be the cause of the irregular deposition of new bone outside the normal limits (*e.g.*, in the skull) which is so characteristic of this disease.

The cases of osteitis deformans enumerated in Table XIII represent all stages of the disease, from those in which apparently only one bone was involved to those in which almost every bone in the body was affected. There is a rough correlation between the

severity of the disease (estimated clinically and from x-ray photographs) and the level of the plasma phosphatase. The most severe cases all had very high values, the milder cases were usually, but not invariably, less high. However, all those shown in this table were definitely cases of Paget's disease. Whether in the very early stages of the disease when, clinically, it is difficult to diagnose, the plasma phosphatase would already be high is uncertain. A high phosphatase, on the other hand, in the overwhelming majority of cases indicates bone lesions of a major type. It is worthy of remark that the phosphatase remains high apparently for the whole duration of the disease—15, 20, 25 years or even more. This may be because there is relatively little excretion of the enzyme from the circulating blood. In the urine of four cases of osteitis with high plasma phosphatase a daily output of 9.8, 18.7, 15.7, and 4.7 units per day of phosphatase was found, or the equivalent of the phosphatase present in 10 to 20 cc. of plasma, whilst in four normal cases under the same conditions 3.9, 4.2, 3.7, and 5.2 units were found, or the amount present in some 30 cc. of plasma. Thus there appears to be no vastly increased excretion of phosphatase in these cases with a high plasma level. A high plasma phosphatase level therefore may not necessarily mean a continuous high rate of leakage of phosphatase from the bones into the blood stream, but a chronic slow diffusion thither at slightly above normal rates.

The work is being pursued further, and the relationship between various factors concerned in calcium and phosphorus metabolism and phosphatase is being investigated experimentally.

SUMMARY.

The phosphatase activity of the blood plasma (or more strictly its ability to hydrolyze sodium β-glycerophosphate) has been determined in a number of normal individuals and a large number of cases of disease. Whilst in normals the phosphatase (expressed in arbitrary units) varies from 0.10 to 0.21, in cases of generalized bone disease—osteitis deformans, generalized osteitis fibrosa, osteomalacia, and rickets (infantile, adolescent, and renal)—the value is very much increased, rising in some cases to above 3 units, which is more than 20 times the normal average value. This abnormality is confined almost exclusively to cases of bone disease. The increase above the normal may be correlated in a

general way with the severity of the disease. It is pointed out that, in addition to the two constituents of the blood which have been known for some years to be affected to a greater or lesser extent in bone diseases, namely the serum calcium and the inorganic phosphate, we have now a third variable—the plasma phosphatase—which suffers still greater changes.

The significance of these findings with regard to the suggested rôle of a phosphatase in bone formation and maintenance, is briefly discussed.

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STUDIES ON GLUTELINS.

VI. THE OPTICAL ROTATION OF THE GLUTELINS OF WHEAT, RYE, BARLEY, MAIZE, AND RICE.*

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In the first paper of this series (1) it was shown that wheat flour contains two glutelins. The elementary composition of these proteins and the distribution of their nitrogen as determined by the Van Slyke method were studied. Characterization of proteins may be made more definite by determining some of their physical constants, such as the isoelectric point, specific rotation, and the so called racemization curve. As reported in our first paper, the same isoelectric points were found for both wheat glutelins (1). In the present paper are given the optical rotations and racemization curves of these proteins. We have also included the optical rotations of the glutelins of rice, corn, rye, and barley. These preparations were soluble in alkali under conditions similar to those given for the α-glutelin of wheat. A satisfactory determination of the optical rotation of the oat glutelin could not be made because of the low solubility of this protein preparation in alkali at the concentration used.

Proteins generally become denatured when in contact with alkali or acid. Because these are the only reagents suitable for dissolving glutelins, it is difficult to establish the optical rotation of this class of proteins as they exist in their native condition. We chose aqueous sodium hydroxide as the solvent agent because alkali solutions are generally used in racemization studies, and because it was found that the figures which we obtained for the optical

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rotations at 20° could be used as the initial points in the racemization curves for the same proteins.

For a satisfactory determination of the optical rotation of a protein when alkali is used as the solvent, at least four factors must be considered: the concentration of the alkali used, the concentration of the protein solution, the temperature, and the length of time the protein is in contact with the alkali. In order to minimize the action of alkali on the protein it is desirable to use a dilute solution of alkali for as short a time as possible at low temperature; and, in order to have a clear solution for good readings, the concentration of protein should be low.

As 0.05 M sodium hydroxide was used in preparing the glutelins, we tried to use the same concentration of alkali when making the determinations of optical activity, but because of the other conditions which had to be maintained, a higher concentration (0.5 M) was necessary, inasmuch as 0.05 M alkali at 20° does not dissolve enough of the glutelins, even after 3 hours, to make possible a reliable reading of the optical rotation.

Under certain conditions alkali has considerable effect on the optical rotation of proteins, as has been shown by Kossel and Weiss (2), Dakin (3), and others. But, under our working conditions, it was found that the specific rotation of the glutelins was only slightly affected, if at all, by 0.5 M NaOH at 20° up to 2 hours.

We determined the racemization curves of the α- and β-glutelins of wheat and of gliadin. With these data and those of Halton (4) for gliadin, we found that the same type of equation, $y = ax^b + c$, was applicable to the three curves. By the use of these equations it was possible to calculate the optical rotations of the proteins for infinitesimal time after exposure to alkali or for all practical purposes for zero time. The calculated values agree well with those found experimentally. It is concluded, therefore, that the values obtained for the specific rotation of other glutelins were not materially affected by the alkali used.

Woodman (5) reported that glutelins prepared from hard and from soft wheats showed different optical rotations. In view of the fact that hard wheat was found to contain two glutelins (1) which showed different optical rotations, it was considered of interest to ascertain whether the α- and β-glutelins of soft wheat

had the same optical activity as the corresponding glutelins of hard wheat. Then, if no differences were observed, Woodman's observations would indicate different proportions of the α- and β-glutelins in hard and in soft wheats. We found, however, no difference in their specific rotations or in their relative proportions in the flour between the glutelins of the hard wheat and those of the soft wheat varieties used in this investigation.

<center>EXPERIMENTAL.</center>

The wheat glutelins used for the work described in this paper were prepared, for the greater part, from a commercial patent wheat flour (Ceresota). For the experiments in which a comparison was made between the glutelins of hard and soft wheat, flour from known varieties of wheat was used (hard wheat, reliance variety; soft wheat, purple straw variety). We are indebted to the Cereal Investigation Laboratory of the Bureau of Agricultural Economics for both the samples of wheat, and for milling the samples in their laboratory experimental mill.

All the glutelin preparations were obtained according to the methods described in previous papers of this series. Approximately 0.4 gm. of the protein was transferred to a 1 inch diameter Pyrex test-tube. The protein was thoroughly wetted with a mixture of 1 cc. of water and 1 cc. of 95 per cent alcohol. While it was agitated constantly with a glass rod, 30 cc. of 0.5 M sodium hydroxide (or 0.05 M) were then added in small portions. The mixture was stirred mechanically until the protein had dissolved and the resulting solution had filtered by suction through a mat of paper pulp which had been just previously washed with sodium hydroxide solution of the same concentration as that used in dissolving the protein. All these operations were carried out at a temperature not higher than 20° and in less than 2 hours. Optical rotations were determined in a 2 dm. tube. A Schmidt and Haensch polariscope set up in a constant temperature room was used. White light filtered through a 2 cm. cell containing 3 per cent potassium bichromate solution was used for illumination. The results of these determinations are given in Tables I, II, and III. Total nitrogen determinations were made on 1 cc. aliquot portions according to Pregl's micro method as modified by Clark and Collip (6). The concentration of protein in the solutions of

TABLE I.

Specific Rotation of Wheat Glutelins in 0.5 M Sodium Hydroxide at 20°.

	Commercial patent flour (Ceresota).		Flour from soft wheat (purple straw).		Flour from hard wheat (reliance).	
	Concentration of glutelin solution.	Specific rotation.	Concentration of glutelin solution.	Specific rotation.	Concentration of glutelin solution.	Specific rotation.
	per cent	*degrees*	*per cent*	*degrees*	*per cent*	*degrees*
α-Glutelin.	0.344	−85.7	0.450	−84.7	0.369	−84.4
	0.458	−83.2	0.592	−87.7	0.556	−84.1
	0.624	−86.1	0.626	−84.4	0.609	−85.4
	0.731	−85.3	0.664	−85.8	0.718	−86.1
	0.990	−85.6				
Average............		−85.1		−85.6		−85.0
β-Glutelin.	0.460*	−75.0	0.653	−76.1	0.682	−77.0
	0.597*	−76.9	0.720	−77.0	0.804	−75.4
	0.628	−77.2				
	1.117	−77.2				
Average............		−76.6		−76.5		−76.2

* Dissolved in 0.05 M sodium hydroxide solution.

TABLE II.

Specific Rotation of the Glutelins of Rye, Barley, Maize, and Rice in 0.5 M Sodium Hydroxide at 20°.

	Concentration of glutelin solution.	Specific rotation		Concentration of glutelin solution.	Specific rotation
	per cent	*degrees*		*per cent*	*degrees*
Glutelin of rye.	0.1998	−82.0	α-Glutelin of corn.	0.237	−73.1
	0.2145	−80.7		0.306	−73.6
	0.2145	−80.7		0.339	−71.5
	0.2340	−81.2		0.505	−72.0
Average............		−81.2	Average............		−72.6
α-Glutelin of barley.	0.146	−112.7	Glutelin of rice.	0.493	−66.7
	0.154	−112.5		0.570	−64.0
	0.250	−110.9		0.657	−68.6
	0.282	−110.4		0.690	−62.7
	0.302	−109.0		0.900	−63.6
Average............		−111.1	Average............		−65.1

the α-glutelin, the β-glutelin, and the gliadin was estimated by multiplying the percentages of nitrogen by the factors 5.8, 6.25, and 5.72, respectively.

For the determination of the so called racemization curves of the wheat proteins samples of each were dissolved in 0.5 M sodium hydroxide solution as described above. Large enough quantities

TABLE III.

Specific Rotation of Wheat Proteins in 0.5 M Sodium Hydroxide at 38°.

Time.	Rotation of gliadin.		Rotation of α-glutelin of wheat.		Rotation of β-glutelin of wheat.	
	Calculated.	Found.	Calculated.	Found.	Calculated.	Found.
hrs.	degrees	degrees	degrees	degrees	degrees	degrees
0	−112.5	−112.7	−90.6	−85.1	−81.3	−76.6
2	−104.6		−83.6	−82.0	−73.6	−70.2
3	−99.8		−79.3		−69.2	
4	−95.6		−76.4	−75.7	−66.1	−68.1
5	−94.1	−92.9	−74.2		−63.8	
9	−87.7	−87.2				
10	−86.5		−67.5		−56.8	
20	−79.2		−61.0		−50.1	
24	−77.3	−77.8	−59.2	−61.6	−48.4	−49.8
48	−70.3	−71.3	−53.0	−57.5	−42.0	−43.3
72	−66.3	−68.6	−48.8		−38.4	
96	−63.4	−65.5	−47.0	−49.1	−35.9	
120	−61.2		−44.9		−34.0	−34.6
144	−59.5		−43.3		−32.5	
168	−58.0	−56.5	−42.0	−42.7	−31.2	
192	−56.8		−40.9	−40.0	−30.1	
216	−55.5		−39.9		−29.0	
240	−54.6	−51.4	−38.0	−37.2	−28.3	
288					−26.8	−25.9

Gliadin = log $(R + 165.1)$ = 2.4434 + (-0.0427) log t. α-Glutelin = log $(R + 158.6)$ = 2.3966 + (-0.0424) log t. β-Glutelin = log $(R + 100)$ = 2.2585 −0.0632 log t.

were used to allow for the removal of several aliquots at different time intervals for optical readings, and for determination of the protein content of the solutions. The clear solutions were placed in an automatically controlled thermostat at 38° and the experiments were considered as starting from that time. The changes occurring showed a progressive and orderly disintegration of the

protein molecule until the optical readings showed little or no change, indicating that an equilibrium had been established. Whether we are dealing here with racemization of the protein molecule as a whole, or with simply a drop in optical activity attributable to the rapid hydrolysis produced by the alkali at 38°, and involving liberation of ammonia and carbon dioxide, remains to be demonstrated by further investigation.

From the data obtained from the racemization studies of the α- and β-glutelins and gliadin, we were able to show that they could all be referred to a hyperbolic curve $y = ax^b + c$. C was deter-

RACEMIZATION   CURVES

Gliadin

α-Glutelin of Wheat

β-Glutelin of Wheat

Specific Rotation

Hours
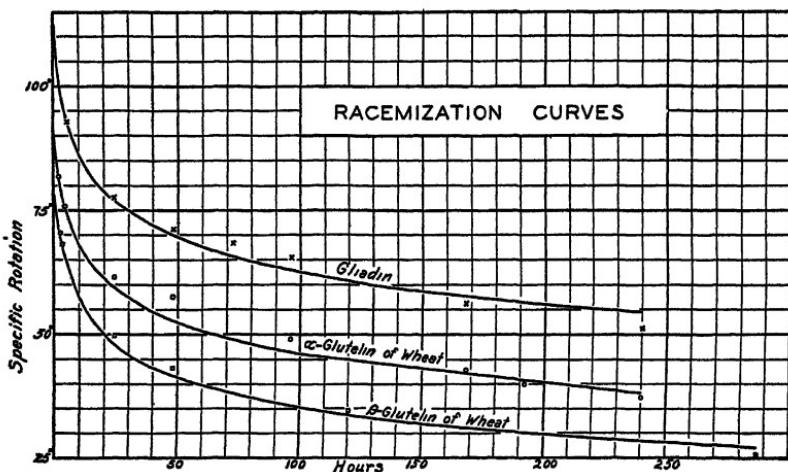
Fig. 1.

mined by plotting specific rotation R against time t, choosing two points on this experimental curve, and the third point so that $t_3 = \sqrt{t_1 t_2}$, and then by calculating R_3. C can be determined from the following equation:

$$C = \frac{R_1 R_2 - R_3^2}{R_1 + R_2 - 2R_3}$$

Rearranging the equation so that it reads $\log (R + C) = \log a + b \log t$, we plotted $(\log t, \log (R - C))$ and obtained a straight line. The calculated values agree well with the observed values. The curve was, therefore, of the type $y = ax^b + c$.

Fig. 1 is a graphical presentation of the calculated specific rotations given in Table III. The specific rotations found experimentally are also indicated at the points marked on Fig. 1.

SUMMARY.

The specific rotation of glutelins prepared from wheat, rye, barley, corn, and rice has been determined.

The α- and β-glutelins of wheat show different optical rotations. No difference was observed in the optical activity of the α- and β-glutelins of hard wheat, and that of the corresponding proteins of soft wheat.

An equation of the type $y = ax^b + c$, proved to be applicable to the racemization curves of gliadin, and to the α- and β-glutelins of wheat.

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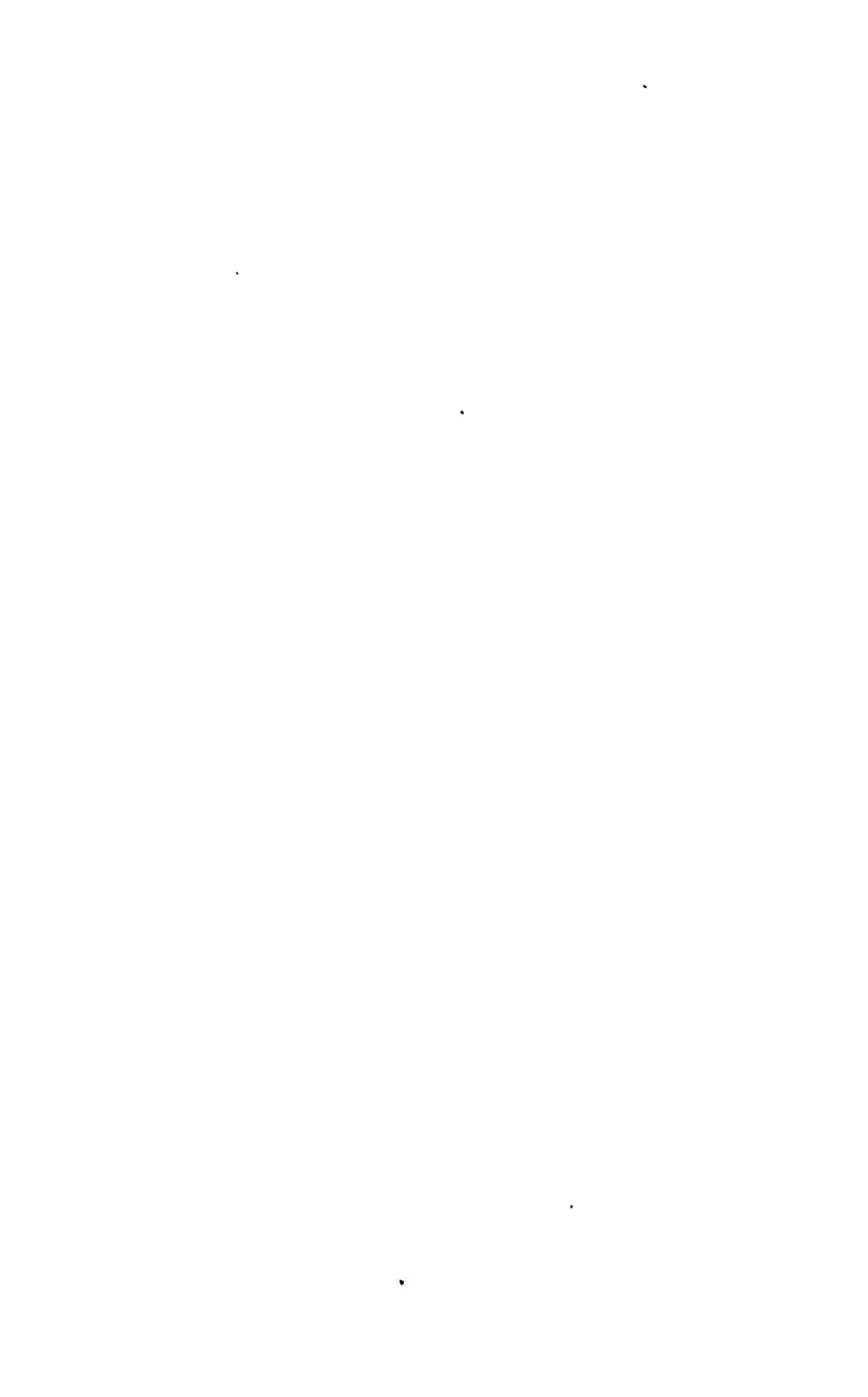
EVIDENCE FOR THE PRESENCE OF A THIRD FACTOR IN THE VITAMIN B COMPLEX OF YEAST.

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Our present day knowledge of the vitamin B complex recognizes several distinct components. Of these, two are so well established that they are definitely designated as the thermolabile, antineuritic factor, B_1, and the thermostable, antipellagric factor, B_2, which is also called the P-P factor of Goldberger and vitamin G. In addition, the existence of other factors has been suggested.

While working on the problem of the separation of the two definitely recognized factors, our interest in a possible third factor of the vitamin B complex was stimulated by the report of Williams and Waterman (1).

Theirs was the first work published which gave evidence for the existence of a third factor in vitamin B. They showed that pigeons fed on a basal diet and the two factors B_1 and B_2 did not grow quite as well and were not in as good general condition as pigeons fed on whole wheat or on the basal diet supplemented by whole brewers' yeast. However, when the residue of yeast from which the factors B_1 and B_2 had been extracted was fed along with these factors plus the basal diet, the birds grew as well as when fed whole yeast or whole wheat. They also showed that this better growth and general condition was not due to vitamins A, D, or E, that the substance is not needed by rats, and that it is thermolabile.

Further evidence for the existence of a third principle in vitamin B has been reported by Hunt (2). He gives data showing that after extraction of the two known factors, B_1 and B_2, the yeast residue still contains some third accessory principle which promotes the growth of rats above that produced by the other two factors alone. He states that this factor is quite thermostable.

Reader (3) has reported experimental results which she interprets as showing that alkaline hydrolysis destroys at least two factors, both thermolabile. These two thermolabile components, B_1 and an unidentified factor, together with the one known thermostable factor, B_2, represent three different principles of vitamin B.

Coward, Key, and Morgan (4) have extracted a thermolabile growth factor from wheat embryo with boiling 90 per cent alcohol and ether and they claim that this factor is distinct from the vitamin B_2 of yeast and that it is not contained in yeast.

Recently, Eddy, Gurin, and Keresztesy (5) have confirmed the findings of Williams and Waterman (1) by showing that the Williams-Waterman vitamin B_3 is not necessary for the proper growth of rats but is needed by pigeons and that it is destroyed by heat. Furthermore, they report that it is labile to alkali.

To summarize: Williams and Waterman (1) have described a *thermolabile* principle which is necessary to the normal growth of pigeons but not to rats and Eddy, Gurin, and Keresztesy (5) have confirmed their work, reporting that their vitamin B_3 is *labile* to both heat and alkali. Hunt (2) has reported a *thermostable* third factor required for the growth of rats. Reader (3) has found a *thermolabile* substance, other than vitamins B_1 and B_2, necessary to the growth of rats. These investigators all obtained their extracts from yeast. Coward, Key, and Morgan (4) extracted from wheat embryo a third principle necessary for the normal growth of rats and *labile* to both heat and alkali. It seems probable that this substance is the same as the principle obtained by Reader (3).

Thus, there have been reported at least three so called third factors (vitamin B_3), each of which exhibits different stability to heat and alkali and is required differently by rats and pigeons. Reading the description of these various third factors in the literature leaves one confused and in doubt as to the nature of any biologically potent factors other than vitamins B_1 and B_2 which may exist in the vitamin B complex.

<div align="center">EXPERIMENTAL.</div>

In general, the procedure used in the experiments reported in this paper is as follows: White rats were fed on a basal diet, deficient in all of the factors of vitamin B contained in yeast, and to this diet were added whole yeast and various fractions of yeast for different groups of rats. In this way the growth curves of rats produced by various fractions fed separately and also various combinations of the fractions fed together were compared with those produced by feeding whole yeast.

<div align="center">*Preparations.*</div>

The method used in the preparation of the various fractions extracted from whole yeast is a combination of various procedures

of extraction used by Williams and Waterman (1), Hunt (2), Levene (6), Seidell (7), Chick and Roscoe (8), and Sherman and Axtmayer (9).

Three preparations were employed: an extract containing the thermolabile, antineuritic factor B_1, an extract containing the thermostable factor B_2, and the residue left after these two factors were extracted from the yeast. They will be referred to as Fraction B_1, Fraction B_2, and Fraction R, respectively.

Methods of Extraction.

Into 10 liters of boiling 50 per cent ethyl alcohol 2500 gm. of dry powdered yeast[1] were stirred and the boiling was continued $\frac{1}{2}$ hour and then the suspension was cooled and covered. It was stirred vigorously several times a day for 1 week and then filtered through muslin in a fruit-press. The residue was treated in a like manner with hot 75 per cent alcohol for 4 days, filtered as before, and the procedure was repeated twice more, once with 90 per cent alcohol for 2 days and finally with 95 per cent alcohol for 1 day. The final residue was pressed as dry as possible and dried in open air. It then weighed 1993 gm., and thus each gm. of the original dry yeast was represented by 0.8 gm. of the residue. This residue was used as Fraction R in the feeding experiments.

All filtrates were combined and evaporated under reduced pressure to a smaller volume. An equal volume of distilled water was added and the liquid was again evaporated. This process was repeated until all of the alcohol was distilled off. The final solution was diluted with distilled water to a volume of 2500 cc., and 500 cc. of this were measured out and labeled "whole yeast extract" and used in the feeding experiments. This whole extract should have contained all of the water-alcohol-soluble vitamin B complex of yeast, and each cc. was equivalent to 1 gm. of the original yeast (2500 cc. of extract from 2500 gm. of yeast).

The remainder of the extract was diluted with water to a volume of 5 liters. After acidification to pH 3.0 with glacial acetic acid, 200 gm. of fullers' earth were added and the mixture was shaken in a machine for 6 hours. Then the suspension was filtered by suction

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[1] All of the yeast used in this work was kindly supplied by the Fleischmann Yeast Company.

through a Buchner funnel and the residue was washed with cold distilled water acidified to pH 3.0 with glacial acetic acid.   The filtrates and washings were combined and treated in the same manner with 200 gm. of fullers' earth.   The combined residues of

Yeast
+ 50 per cent alcohol

Residue
+ 75 per cent
alcohol

Residue
+ 90 per cent
alcohol

Combined filtrates
+ 400 gm. fullers'
earth in two parts

Residue
+ 95 per cent
alcohol

Residue
(washed and
dried in air)

Combined filtrates
and washings
+ water
(concentrated
in vacuo)
+ water to
500 cc.

Residue
(dried in
air)

Fraction B_1.   Fraction B_2.   Fraction R.
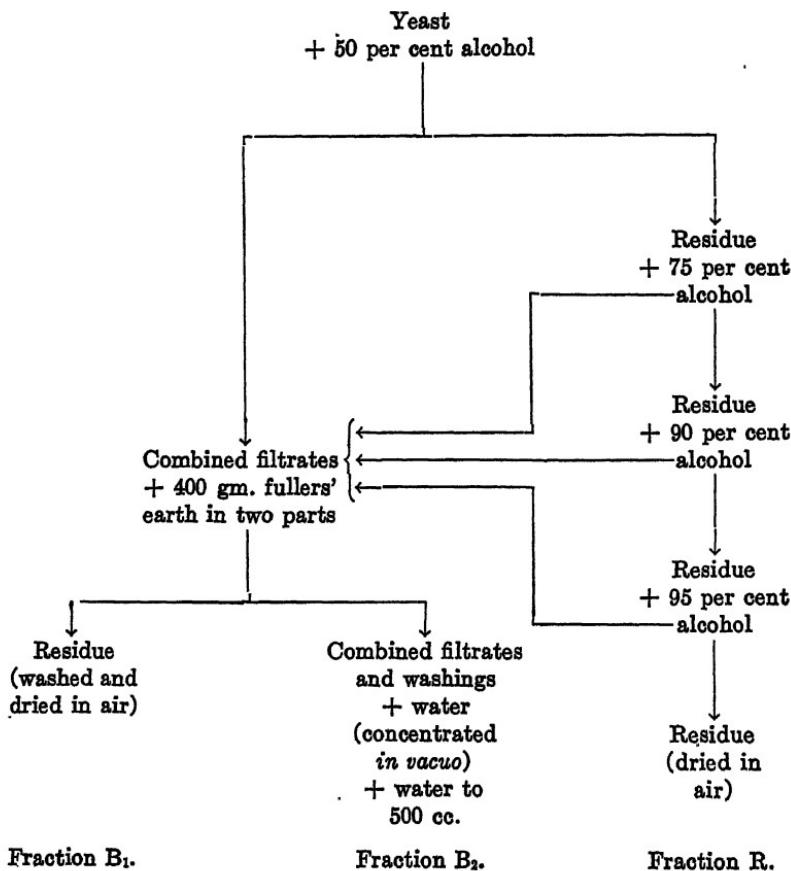
Steps in preparation of yeast fractions.

fullers' earth were dried in open air.   This fraction should contain the thermolabile, antineuritic factor B_1.   Each gm. represented 5 gm. of the original yeast.

The combined filtrates and washings of this last procedure were concentrated under reduced pressure over a water bath to a volume

of about 100 cc. and an equal volume of water was added. This procedure was repeated until the solution was neutral to phenol red. Distilled water was then added to bring the total volume to 500 cc. This fraction should contain the thermostable, antipellagric factor B_2. Each cc. represented 4 gm. of the original yeast.

In the accompanying diagram is an outline of the procedure described above of extraction of the yeast and the preparation of the three fractions.

Technique of Animal Experiments.

In all of the experiments healthy albino rats of 4 weeks of age were used. They were kept in individual cages and were fed separately. The cages were the cylindrical type used by Osborne and Mendel as described by Ferry (10), and were made of half inch galvanized iron screening. Instead of using them as suggested by Osborne and Mendel, we inverted them and fastened a lid of screening over the upper, open end. The cages were then set in large, flat pans and the floor of each cage was held about one inch above the bottom of its pan with a galvanized sheet iron hoop so that all excreta fell through the false floor and were inaccessible to the rats. Each cage was cleaned thoroughly each week and the animals were kept in a clean environment in a room ranging in temperature from 18–21°.

In each individual cage there were fastened two small jelly glasses so that they could not be tipped over by the rats. One glass was kept filled with clean fresh water. The other glass was fitted with a tin cover in the center of which was cut a round hole just large enough to admit the rat's head. This prevented the scattering and consequent waste of food by the rats.

The basal diet was deficient in vitamin B but contained all of the other vitamins essential to the normal growth of rats. The diet used was that employed by Sherman and Spohn (11). The formula is as follows:

	per cent
Extracted casein	18
Corn-starch	68
Butter fat	8
Osborne and Mendel salt mixture.*	4
Cod liver oil	2

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

Any possible vitamin B in the casein was extracted by a modification of the method of Sherman and Spohn (11). Commercial casein was poured into hot 65 per cent alcohol and stirred for 6 hours. Next, a large quantity of tap water was added and the mixture was stirred for 1 hour and then allowed to stand overnight. The supernatant fluid was decanted off, more water was added, and the mixture was stirred as before. This procedure was repeated four times and the casein was finally washed with 80 per cent alcohol, pressed as dry as possible in a fruit-press, and thoroughly dried in open air.

For 2 weeks immediately preceding the experimental period, each rat was fed on the basal diet alone. This was for the purpose of eliminating any vitamin B that the rat might have stored in its body. During the experimental period, each animal was fed the basal diet *ad libitum* and the proper vitamin B fraction or combination of fractions. Each morning the food containers were removed from the cages and kept out until about noon so that the rats would get hungry. The fractions which were dry (Fractions B_1 and R) were then fed in proper amount mixed with a small amount of basal diet in the bottom of the container. Fraction B_2 was fed by mixing the proper amount with enough basal diet to absorb it and form a small pellet. Being hungry, the animals immediately ate these small amounts of food without waste. Only after they had eaten all of this food were they given additional basal diet.

Once each week a careful record was made of the weight and general condition of each rat and any symptoms which might have developed during the past week.

Study of the Various Yeast Fractions.

Forty-two animals were divided into eight groups of four animals each and two groups of five animals each. The rats were fed individual fractions of the yeast extract and combinations of these fractions as indicated in Table I. In discussing the results of these feeding experiments, the amount of original yeast represented by a given dose of a fraction (B_1, B_2, or R) will be designated by the term "gm. yeast equivalent." Thus, since 1 gm. of Fraction B_1 represents 5 gm. of the original yeast, this dose (1 gm.) would be designated as 5 gm. yeast equivalents of Fraction B_1.

It is obvious that the first five are control groups for the purpose of determining the potency and biologic properties of the individual fractions.   The last five groups are experimental and by them is determined whether or not the residue (Fraction R) contains any principle which will supplement the two known factors of vitamin B.

At the end of the experimental period of 14 to 16 weeks or at any time that definite and marked symptoms of polyneuritis or inani-

TABLE I.

Nature and Amount of Yeast or Fractions Thereof Fed to Rats of Different Experimental Groups.

Group No.	Yeast or fraction.	Amount fed per rat per day.	Gm. yeast equivalent per rat per day.
		gm.	*gm.*
I	None.	None.	None.
II	Whole yeast.	1	1
III	B_1.	0.4	2
IV	B_2.	0.5	2
V	R.	1.6	2
VI	Whole yeast extract.	1	1
VII*	Same + R.	1 + 0.8	1 (of each).
		2 + 1.6	2 " "
VIII*	$B_1 + B_2$.	0.2 + 0.25	1 " "
		0.4 + 0.5	2 " "
IX	" + "	0.8 + 1	4 " "
X	" + " + R.	0.2 + 0.25 + 0.8	1 " "

* Two of the rats in each of these groups were fed 1 gm. yeast equivalent of each fraction and three animals were fed 2 gm. yeast equivalents.

tion developed the rats were killed by ether and the brain and cervical cord were removed for histological study.   The results of this investigation will be presented in a subsequent report.

Charts I to III show the growth curves of all the rats used in the experiments.

The animals on the basal diet alone were carried as negative controls and show the inadequacy of this diet to promote growth (Group I, Chart I).   One animal, Rat 101, died with polyneuritic symptoms in 11½ weeks.   The others lived 16 weeks, but only one other rat developed symptoms suggestive of polyneuritis.   All four

animals developed lesions of the eyelids and nose characterized by redness, bleeding, and loss of hair, similar to those reported by Sherman and Bourquin (12) as due to the deficiency of vitamin B_2 or G.

With one exception, the animals fed whole yeast (Group II, Chart I) showed the expected excellent general condition and nor-
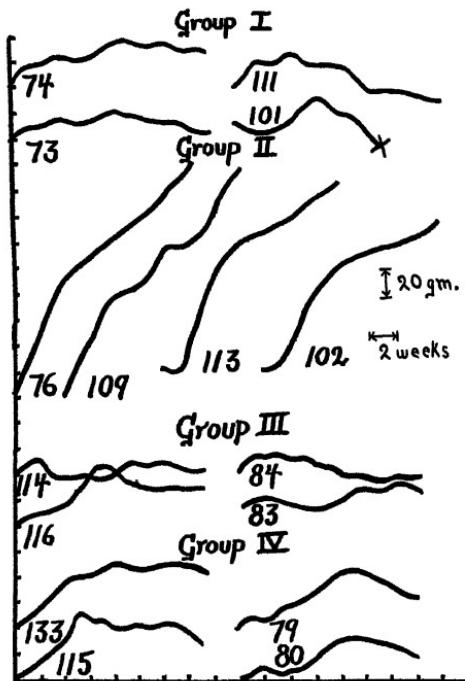
CHART I. Growth curves of rats showing the effect of additions to the basal diet of various yeast fractions: no addition (Group I), whole yeast (Group II), Fraction B_1 (Group III), and Fraction B_2 (Group IV).

mal growth. One rat, No. 102, did not gain well and about 1 week after the termination of the experiment developed symptoms of pneumonia and died. Undoubtedly, this explains its failure to grow normally.

The rats fed with the antineuritic Fraction B_1 (Group III, Chart I) failed to grow, lost some weight, and died, but in none of

them did any symptoms of polyneuritis develop.   They all developed red, bleeding lesions of the eyelids and nose.

The animals receiving the antipellagric Fraction B$_2$ (Group IV, Chart I) gained some weight at first but later lost a little and in two cases (Rats 79 and 80) developed a few symptoms suggesting polyneuritis.   These two animals were killed as soon as death became imminent in order that the central nervous system might
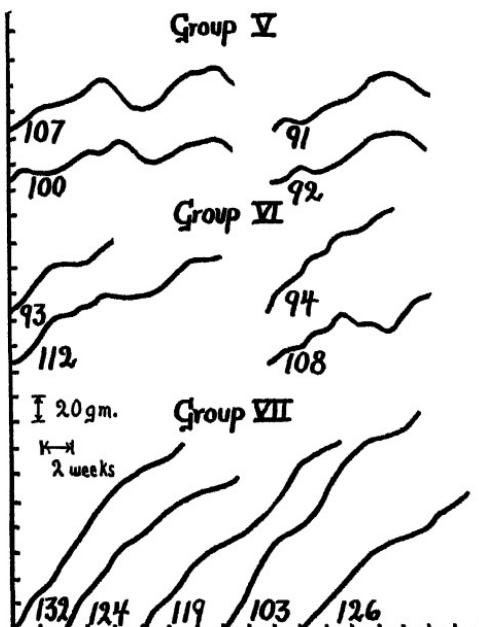
CHART II.   Growth curves of rats showing the effect of addition to the basal diet of various yeast fractions: residue, Fraction R (Group V), whole yeast extract (Group VI), and yeast extract plus residue, Fraction R (Group VII).

be studied.   None of the animals developed the red, bleeding lesions of the eyelids and nose which occurred in the animals fed a diet deficient in vitamin B$_2$.   These results show that Fraction B$_2$ contains an adequate amount of the antipellagric vitamin B$_2$.

The residue (Fraction R) which was fed to the rats of Group V (Chart II) failed to promote much growth, although the curves are not as flat as those of the animals of Group I (no yeast fraction).

All of the rats developed the lesions of the eyelids and nose described above but none of them died with true polyneuritic symptoms nor from inanition during the period of experimentation (15 weeks).   The fact that no symptoms of polyneuritis developed in these rats indicates that the Fraction R was free of vitamin B_1. This argument is supported by considerable evidence in the literature (cf., for example, Kinnersley, Peters, and Reader (13), Chick
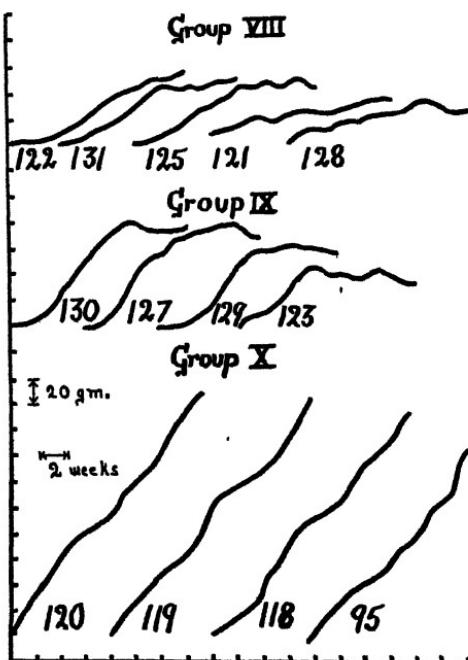
CHART III.   Growth curves of rats showing the effect of additions to the basal diet of various yeast fractions: B_1 and B_2 (Groups VIII and IX) and B_1 and B_2 plus residue, Fraction R (Group X).

and Roscoe (8), Drummond (14), Evans and Lepkovsky (15), and Sandels (16)) showing that when the diet contains no antineuritic vitamin B_1 rats are not as apt to develop symptoms of polyneuritis as when traces of this vitamin are present.

The growth curves of the rats in Group VI (Chart II) (whole yeast extract) are better than those in any preceding group except Group II (whole yeast) but, even so, are not entirely normal.

The animals developed no symptoms of any type. The growth curves simulate those of the animals fed various amounts of Fractions B_1 plus B_2 (Groups VIII and IX, Chart III).

When residue was added to the whole yeast extract and fed to the animals in Group VII (Chart II) the resulting growth was normal. The two rats (Nos. 103 and 119) fed on 1 gm. yeast equivalent each of whole yeast extract and Fraction R (residue) per rat per day grew as well as those (Rats 124, 126, and 132) receiving 2 gm. yeast equivalents of each.

The growth of the rats fed Fractions B_1 plus B_2 (Groups VIII and IX, Chart III) was not normal but was much better than that of the animals fed on any of the Fractions B_1, B_2, or R, separately (Groups III, IV, and V). The feeding of 4 gm. yeast equivalents each of Fractions B_1 and B_2 (0.8 and 1 gm., respectively) per rat per day (Group IX) instead of 2 gm. yeast equivalents (0.4 and 0.5 gm., respectively) per rat per day (Rats 125, 128, and 131, Group VIII) did not improve the resulting growth over that in rats (Nos. 121 and 122, Group VIII) which received only 1 gm. yeast equivalent (0.2 and 0.25 gm., respectively) daily.

The last group (Group X, Chart III) in which the animals were fed only 1 gm. yeast equivalent per rat per day of each of the Fractions B_1, B_2, and R shows curves which are absolutely normal as compared with those in Group II where whole yeast was fed.

<center>DISCUSSION.</center>

The results obtained in Groups I to V need little comment. Groups I and II, respectively, are the negative and positive control groups, and Groups III, IV, and V show that none of the individual fractions is adequate for normal growth.

The results with the animals of Group VI, in which the whole extract of yeast was fed, show that the growth curves of these rats are not normal in any case. This means that 1 gm. yeast equivalent of the alcohol-aqueous extract of yeast is not equivalent to 1 gm. of whole yeast in its potency to promote the growth of rats. This may be explained in one of two ways: Either the extraction has not removed all of the water-soluble vitamins B_1 and B_2 from the yeast or there remains in the residue a *water-insoluble* growth principle. We believe that the results obtained in these experiments favor the latter explanation.

The premises upon which our argument is based are two important facts obtained from the experimental results.

First, 1 gm. yeast equivalent of whole yeast extract (Group VI) or 1 gm. yeast equivalent each of Fractions B_1 and B_2 per rat per day (Group VIII) promotes growth to some extent but not as well as does whole yeast (Group II). Twice the daily dose of Fractions B_1 and B_2 (Rats 125, 128, and 131, Group VIII) or even 4 times the original daily dose (Group IX) promotes the growth of the animals little, if any, more than does the single daily dose.

Second, as little as 1 gm. yeast equivalent each of the Fractions B_1, B_2, and R combined per rat per day produces normal growth (Group X) as does 1 gm. yeast equivalent each of Fraction R and whole yeast extract (Group VII).

Consequently, since even 4 gm. yeast equivalents each of Fractions B_1 and B_2 per rat per day are not adequate to produce normal growth, whereas the addition of 1 gm. yeast equivalent of Fraction R per rat per day to 1 gm. yeast equivalent each of Fractions B_1 and B_2 is sufficient, the residue, Fraction R, cannot owe its potency to vitamins B_1 and B_2. Hence, the residue, Fraction R, contains some third factor which is required for the optimum growth of rats and which is *qualitatively* different from vitamins B_1 and B_2.

That this accessory growth-promoting substance left in the yeast residue is neither vitamin A nor vitamin D is shown by the fact that the basal diet contains adequate amounts of each in the butter fat and cod liver oil. Furthermore, it cannot be of mineral nature since an optimum amount of Osborne and Mendel salt mixture was incorporated in the basal diet.

This third factor cannot be identified as that extracted from wheat embryo by Coward, Key, and Morgan (4) or obtained from yeast by Reader (3), for these workers found thermolabile principles. Furthermore, it cannot be the same as that of Williams and Waterman (1) and Eddy, Gurin, and Keresztesy (5), who report that rats do not require their third factor. It is quite probable that our third factor is identical with that obtained by Hunt (2), inasmuch as both are thermostable and required by rats.

The writers feel that their experiments supplement the investigations of Hunt (2) by showing with proper controls that the principle contained in the residue is distinctly different from either vitamins B_1 or B_2. In fact, there is not yet adequate evidence for

assuming that this third principle is even a part of the vitamin B complex. Work on this substance, which we are not yet ready to report, suggests that it is a distinct principle, for it seems to be absolutely insoluble in hot or cold water, alcohol, or acids. No successful method for extracting this third factor from yeast residue has yet been reported in the literature.

SUMMARY.

We believe that the experiments described above justify the following conclusions:

1. The factors B_1 and B_2 of vitamin B, when supplementing a vitamin B-deficient diet, are not alone sufficient to promote the optimum growth and health of albino rats.

2. After extraction of the water-alcohol-soluble fractions from yeast, the residue contains a growth-promoting principle which is necessary for the maintenance of normal growth of rats.

3. This third factor is qualitatively distinct from either factors B_1 or B_2 of the vitamin B complex. It is insoluble in water and alcohol and is thermostable.

4. In the recent literature there are reports of at least three different third factors occurring in the vitamin B complex. We have obtained from yeast a principle which supplements vitamins B_1 and B_2 and seems to be similar to the factor of Hunt but differs from all of the others described.

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STUDIES IN THE PHYSICAL CHEMISTRY OF MUSCLE GLOBULIN.*

II. ON SOME PHYSICOCHEMICAL PROPERTIES OF MUSCLE GLOBULIN (MYOSIN).

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INTRODUCTION.

The globulin of muscle was studied in the late nineteenth century by a number of investigators, especially Danilewsky (11), Halliburton (18), and von Fürth (14, 15). In recent years, Weber (32) has made important contributions to the chemistry of this protein (myosin) and its quantitative characterization. Its behavior is still, however, very inadequately known. There are, indeed, peculiar difficulties connected with the extraction, purification, and exact study of tissue proteins, which do not exist with regard to blood, white of egg, and the resting seeds of plants, which have been the source of the proteins whose character is at present most accurately known. Oxidative and other enzymatic processes may alter the protein profoundly during extraction from an active tissue such as muscle. The structure of the cell must be shattered to let the protein out, and in the process, changes in the protein, of uncertain character, may take place. Even after extraction the protein is particularly unstable unless kept under certain well defined conditions.

The need for more knowledge in this field is, however, steadily increasing. Knowledge of the energy-liberating reactions in muscle has made enormous strides in the past 20 years (Meyerhof

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* The preceding paper in this series is by W. T. Salter (29). Muscle globulin has also been studied in this laboratory by Dr. F. F. Heyroth (unpublished experiments in the files of the laboratory).

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(25)), and far surpasses our knowledge of the machinery which they set in motion. It seems beyond doubt that the proteins play a large—if not the largest—part in this machinery. It is to be expected that the physicochemical properties of the isolated protein will be found intimately related to its function within the muscle fiber. The data of this paper, although necessarily incomplete and inadequate, are presented as a contribution to this problem.[1]

Preparation.[2]

Two different sources of material were used in the studies reported here; the cheek and jaw muscle of cows, and the hind leg muscles of rabbits. The method of preparation is in principle the same as that employed by earlier observers (Danilewsky (11), Halliburton (18), von Fürth (14), and Howe (22)), the fresh finely ground muscle being extracted by faintly alkaline salt solutions of moderate concentration. When cows were used, the muscle was obtained at the slaughter-house as rapidly as possible after the death of the animal, ground up immediately (fat and tendon being removed as far as possible) and stirred into bottles containing the salt solution used for extraction. Much blood is obtained with the muscle protein, but the blood proteins can be completely removed by methods discussed below. The preparation made from cows is referred to in what follows as cow muscle globulin (or simply C.M.G. with the number of the preparation attached).

The rabbits studied were anesthetized with ether or killed by a blow on the head, and the lower extremity perfused with isotonic potassium phosphate solutions (pH 7.8) by means of cannulæ in the abdominal aorta and the inferior vena cava, until the fluid coming from the vein was colorless. (This procedure is very similar to that employed by Weber (32) as the first step in the preparation of myogen and myosin.) The hind legs were then skinned, the

[1] Certain highly distinctive characteristics of the protein are studied at length in two following papers by von Muralt and Edsall.

[2] Much of the method of preparation here described was worked out by Dr. E. J. Cohn and Dr. F. F. Heyroth in this laboratory, in 1926–27. They used exclusively phosphates in the extraction; the use of chloride-phosphate mixtures was introduced later.

muscles removed, freed from fat and tendon, ground fine in a meat grinder, and stirred rapidly into cold salt solution. The preparations made from rabbits will be called simply rabbit muscle globulin (R.M.G.).

Certain precautions must be observed if the characteristic protein is to be obtained in considerable amounts and undenatured. The muscle must be obtained as fresh as possible (within 30—preferably 20—minutes after the death of the animal), and its structure well broken up. The whole system should be kept as cold as possible (the extracting salt solution is cooled to 4°, or below, before use). The salt solution should be slightly alkaline (pH 7 to 8.5) and well buffered. The pH should be watched carefully during extraction, and small amounts of weak alkali added if necessary. Steady (but not violent) mechanical stirring is carried on throughout the extraction.

The need for some of these precautions deserves a little discussion. The importance of obtaining fresh muscles, very soon after death, is emphasized by the work of Saxl (30). From fresh muscle, finely minced, and repeatedly extracted in the cold, Saxl could extract nearly 90 per cent of the total protein in a soluble form. After rigor had set in, 70 per cent of the muscle protein remained undissolved, even after repeated extraction with 10 per cent ammonium chloride solution. The recent interesting work of Deuticke (12) and of Hensay (19) shows that pronounced alterations in the solubility of muscle proteins take place within the first half hour in chopped muscle. The importance of breaking up the muscle structure as completely as possible is obvious; the increased exposure of surface and the destruction of impermeable cell membrane facilitates the extraction of protein, and enables buffering substances to neutralize lactic and other acids more readily.

On the acid side of pH 6.0 the solubility of the protein is extremely low at all salt concentrations, hence the extracting medium should be kept alkaline to this point. In the studies here reported, K_2HPO_4(pH about 8.5) was used to buffer the acids formed in the muscle, and the pH at the end of the extraction was generally 7.0 to 7.4. The pH was tested colorimetrically (with brom-thymol blue, cresol red, and phenol red) at frequent intervals during the extraction, and more K_2HPO_4 added if the pH fell below 7.0. The addition of strong alkali, even very dilute,

is dangerous to the protein, which is profoundly altered by a pH slightly alkaline to 10.[3]   This danger can be avoided by using K_2HPO_4 to buffer the acids present, so that the system never becomes alkaline to pH 8.5.

At the beginning of these experiments, phosphate buffer solutions were used for the extraction, following a procedure very similar to that of Howe (22).   Later Dr. Howe informed us (personal communication) that he had obtained more effective extraction with potassium chloride than with phosphate—an observation which we have confirmed.[4]   The procedure finally adopted was to use potassium chloride (1.2 M) as an extracting medium, with the addition of K_2HPO_4 (0.05 M) and KH_2PO_4 (0.01 M) to buffer the solution.   The total ionic strength is therefore between 1.3 and 1.4.

The amount of meat used should not be too large in proportion to the volume of the extracting medium.   If the chopped meat is in equilibrium with a large volume of solution, extraction is facilitated, and the protein more rapidly removed from the muscle fiber.   About 1 volume of meat to 3 or 4 of solvent was generally used in these experiments.   If less solvent is used, protein preparations with different properties are sometimes obtained, with lower viscosity, less light-scattering power, and higher solubility. It appears probable that muscle proteins may be altered during extraction by the activity of the enzymes present, and especially by oxidations.   Hopkins (21) has shown that oxidation of protein with glutathione takes place only if the protein itself possesses free sulfhydryl groups, and muscle proteins are known to be more rich than most in these.   Rapid extraction of the protein is probably the best method of minimizing this danger.[5]   Steady mechanical stirring and the presence of a large volume of extracting fluid were therefore employed to bring this about.

The extract is stirred steadily by a mechanical stirrer for 1½ to 2 hours; the meat is then strained off with cheese-cloth, and the

[3] The evidence for this statement is presented in a following paper by von Muralt and Edsall.

[4] Hensay has found that potassium salts in general extract muscle proteins more effectively than do the corresponding sodium salts.

[5] The addition of cyanide to the extracting medium might also be of value, but this has not yet been tried.

fluid filtered with suction through a layer of filter paper pulp about 1 cm. thick. The filtrate is water-clear. If cow protein is used, the fluid is a deep red color from the hemoglobin present; if a perfused rabbit has been used, the fluid is pale yellow.

KH_2PO_4 is now added with stirring to the filtrate, until the pH (measured colorimetrically) is about 7. The filtrate is then diluted 10- to 20-fold with cold distilled water to which a trace of phosphate buffer has been added.[6] (Rabbit muscle globulin which appears to be somewhat more soluble than cow muscle globulin in dilute salt solutions, may require a 20- to 30-fold dilution.) A heavy cloudy precipitate forms which settles to the bottom. The supernatant fluid is siphoned off, and the precipitate concentrated by centrifugation, preferably in the cold. The precipitate readily redissolves on the addition of moderate quantities of any neutral salt (potassium phosphate buffers were generally used in these experiments). Higher concentrations of neutral salts readily precipitate the protein. It is, for instance, completely precipitated by potassium phosphates at ionic strength 3.6 pH 7.4 at 0°; and by lower ionic strengths at higher temperatures. At pH 6.8 and 0° it is completely precipitated at ionic strength 3.0. (The total molal concentration of phosphate in either of the solutions mentioned is about 1.2.) The precipitate thus formed may be separated by centrifugation and redissolved by the addition of water. It is advisable to add' only just enough salt solution to precipitate the protein. If more is added, centrifugation becomes very slow and difficult, owing largely to the greater density of the solution, relative to the particles of precipitate. There is also greater danger of denaturing the protein if the ionic strength is excessively high.

The processes of precipitation by increasing or decreasing the ionic strength may be repeated as often as is desired. Protein used for solubility measurements was generally precipitated six times—three times by dilution and three times by "salting out."

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[6] The trace of salt is added as a precaution, some proteins (*e.g.* edestin, Osborne (26)) being altered in their properties by contact with pure distilled water. No harm appears to have occurred, however, in a number of preparations here studied in which this precaution was omitted. It is probable that the altered protein is formed only slowly, as the precipitated protein is allowed to *stand* with distilled water.

The protein should never stand very long in the precipitated condition. A solution which has been precipitated by dilution can safely stand overnight in the cold room, but by the end of 24 hours some of the precipitate has generally become permanently insoluble. Material which has been precipitated by high ionic strength is often very unstable (especially at room temperature) and should not be left undissolved for more than an hour or so (although sometimes the precipitate formed by "salting out" is apparently quite stable).

Cow muscle globulin is completely insoluble in salt solutions of ionic strength 0.10, near pH 7 while all the blood proteins (including fibrinogen, which has recently been studied in this laboratory by Dr. Marcel Florkin) are soluble at this ionic strength. Myogen (see Weber (32, 34)) is highly soluble even in pure water. Consequently repeated precipitation of muscle globulin at this salt concentration should leave all other proteins in solution. Similarly, muscle globulin "salts out" at a lower ionic strength than myogen or any of the blood proteins except fibrinogen. By the use of both methods of precipitation, therefore, it should be possible to purify muscle globulin effectively. The data given below, however, indicate that even the purest preparations thus far obtained do not consist of one single chemical individual.

Appearance of Protein.

Muscle globulin, purified by the method described above, is a viscous liquid, golden yellow by transmitted light, with a high light-scattering power, so that it appears blue even by reflected daylight, and shows an extremely intense Tyndall cone in a concentrated light beam. The character of this light scattering is unaltered by repeated precipitation and resolution of the protein.

Crystals of muscle globulin, visible to the naked eye or under the microscope, have never been obtained by any of the methods used in this laboratory; nor has any mention of a crystalline muscle globulin been found in the writings of earlier observers. When precipitated, either by increase or decrease of the ionic strength, it settles out in the form of fine gel-like micellar particles, which concentrate to a thick jelly-like mass on centrifugation. During resolution, the micellar form of the particles is still more striking. As solution proceeds, the micelles gradually disappear, and a solu-

tion is formed, which does not contain any particle visible either in the polarizing microscope or in the ultramicroscope.

Point of Minimum Acid- and Base-Binding and the Isoelectric Point of Muscle Globulin.

Weber (32) has measured microscopically the electrical migration of precipitated myosin particles[7] from the frog and rabbit, and reports an isoelectric point close to 5.1. He has also measured the swelling of muscle residue from which the juice had been squeezed out, and found a minimum of swelling likewise at pH 5.1.

Salter (29) studied the behavior of muscle globulin from another angle. He determined the point at which the protein appeared to be uncombined by washing precipitated cow muscle globulin repeatedly with CO_2-free distilled water, and measuring colorimetrically the pH of water saturated with the protein. The pH so measured came invariably to 6.2 to 6.6, agreeing with the flocculation maximum which Collip (10) had found in the same zone for muscle proteins. These measurements have been repeated a number of times in these studies, with muscle globulin from both the rabbit and the cow. The indicators used were brom-cresol green, brom-thymol blue, and chlor-phenol red, the color of the protein-saturated water being matched against known buffer mixtures. The results were identical with those of Salter.

This procedure might perhaps be open to objection, since the solubility of muscle globulin in pure water is almost negligibly low. Water saturated with the protein has therefore almost no buffering power, and the addition of the indicator itself might conceivably alter the pH appreciably in such an unbuffered solution (Clark (5) p. 190). The problem of the point of minimum combination with acid or base was therefore studied also by another method, which has previously been used by a number of investigators. Suspensions of the protein were mixed with very dilute buffer solutions, and the resulting shift in the pH of the solution was observed.

The protocol of one such experiment follows.

40 cc. of Rabbit Muscle Globulin II were precipitated by dilution,

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[7] Weber's myosin appears to be identical with our muscle globulin. See the discussion at the end of this paper.

and washed repeatedly with CO_2-free distilled water. 5 cc. of the centrifuged protein precipitate were taken after the sixth and seventh washing and stirred rapidly into 10 cc. of 0.001 N acetate buffer, pH 5.7. The mixture was centrifuged a few minutes in a well stoppered test-tube before adding the indicator (to prevent any direct reaction between indicator and suspended protein) and 1 drop of chlor-phenol red was added to 5 cc. of the supernatant fluid. The color corresponded to that of a standard buffer at pH 6.0, indicating a shift of 0.3 of a pH unit toward the alkaline side.

As a control, 5 cc. of the CO_2-free distilled water used in the washing were added to 10 cc. of the 0.001 N acetate buffer, centrifuged in the same manner, and again 1 drop of indicator added to 5 cc. of the liquid. The color of this control corresponded to pH 5.7 (*i.e.* that of the original buffer).

The residual undissolved protein could be readily redissolved in dilute salt solution.

These experiments have been carried out a number of times on both cow and rabbit muscle globulin, always with similar results. It therefore appears that uncombined muscle globulin treated with buffer at pH 5.7 reacts with the acid of the buffer solution. The ratio salt:acid in the acetate buffer is therefore increased and the H^+ ion activity diminished. Therefore muscle globulin functions as a base at pH 5.7 and the point at which it binds neither acid nor base appears to lie alkaline to pH 6.0.

These experiments, like those of Salter, give information as to the pH of minimum acid- or base-binding for muscle globulin. They do not necessarily yield the isoelectric point, which strictly speaking can only be established by cataphoretic measurements.

To be entirely satisfactory, cataphoretic measurements must be carried out on the protein in solution, and in the nearly complete absence of salt.[8] Theoretically there is no adequate reason to assume that the isoelectric point of the precipitated protein is identical with that of the dissolved substance. The two are in fact almost identical for many proteins, but the relation remains empirical.

[8] See Weber's excellent discussion (32) of the influence of salt on the isoelectric point of myogen and myosin, and his theoretical treatment of the same problem (33). See also Simms (31) for the influence of ionic strength on the isoelectric point of simple ampholytes.

Muscle globulin, as Salter (29) has shown, is insoluble in the absence of salt over a wide pH range. The isoelectric points of proteins which behave similarly in this respect are often difficult to determine. Cohn ((6) p. 401) in discussing the data concerning edestin and the prolamins concludes,

"These conflicting results render it certain that the isoelectric point is not easily determined in the case of proteins which form a series of insoluble compounds. The interpretation of their behavior demands a knowledge not only of the reaction at which the neutral protein exists, but of the reactions and compositions of the several insoluble compounds."

Weber's measurements, therefore, while entitled to great weight, cannot be said to have settled the problem. His measurements were made on the precipitated protein (necessarily, for myosin at pH .5.1 is not appreciably soluble even in the presence of salt) and are affected with the uncertainty discussed above.[9]

In short, the behavior of muscle globulin (myosin) has been studied by two quite different methods. The theory of the relation between the isoelectric point of a protein, and the point at which its acid- and base-binding is at a minimum, has not yet been fully developed. The assumption that the two are identical is valid only as a first approximation. In the absence of an adequate theory, we may for the present remain content to present the data thus far obtained; the results of Weber giving pH 5.1 as the isoelectric point of the precipitated protein, the results of Salter and Edsall giving pH 6.2 to 6.6 as the zone of minimal acid- and base-binding.

Solubility.[10]

In the absence of salt, and even in the presence of salt at low ionic strengths, muscle globulin is almost completely insoluble.

[9] Dr. Weber (personal communication) writes that he has confirmed the measurements made in this laboratory on the acid- and base-binding of the washed protein, and also confirms his own earlier measurements on cataphoresis. Experimentally, therefore, Weber's results are apparently in complete accord with those reported here. Weber suggests that even carefully washed myosin may still contain some of the alkali salt of the protein; a possibility that deserves further investigation.

[10] A number of the first solubility determinations on muscle globulin (unpublished) were made by Dr. F. F. Heyroth in this laboratory; a few earlier determinations (unpublished) were also carried out by Dr. W. T. Salter.

Salter (29) states, "this fraction dissolved in distilled water, at 25°C., less than 0.025 gram (4 mg. protein nitrogen) per liter." In the studies reported here, solubilities as low as this were found even with ionic strengths as high as 0.20.

Methods.

Almost all solubility determinations were carried out at 0°, as the undissolved protein is readily denatured at higher temperatures. The purified protein was washed free of salt by repeated precipitation and centrifugation with nearly salt-free water, or else the salt present in the protein solution was directly determined by analyses. To determine phosphates, the protein was precipitated by trichloroacetic acid, and the phosphate determined by the method of Fiske and Subbarow (13). Phosphate buffer mixtures of known pH and ionic strength (Cohn (7)) were then added to the protein solution, in proportions calculated to bring the whole system to a definite pH and ionic strength. Phosphate analyses were carried out at the end of the experiment on each sample to check the calculated value. The solutions were placed in 250 cc. or 500 cc. centrifuge flasks, which were placed in holders, and plunged nearly to their necks in an ice bath in a cold room at 4°. Slow stirring was carried out by glass stirrers turned by an electric motor. After periods varying from 4 to 48 hours, the flasks were removed, and the precipitated protein separated by filtration or centrifugation. Both these processes had to be carried out at 0°. The filter funnels were surrounded with ice water during filtration; and the centrifugation was carried out in test-tubes (capacity 50 cc.) suitably held in place and surrounded by crushed ice in large centrifuge cups. In the cold room centrifugation could be carried on for about 20 minutes without melting all the ice. This time usually sufficed to separate the precipitate from a clear supernatant fluid.[11] The precipitate is tested for solubility in neutral salts of a concentration where the protein normally dissolves. If it is not still largely soluble, the determination is rejected.

[11] If the concentration of dissolved protein is very low (0.1 per cent or less) filtration can be employed; but above this concentration filtration is impossibly slow at 0°, and the filter paper soon becomes clogged with a layer of gelatinized protein. Even filtration with suction is very ineffective.

A suitable aliquot of the supernatant fluid is then taken, digested, and analyzed for nitrogen by the Kjeldahl method, and the result calculated in terms of mg. of protein nitrogen dissolved per liter.

As the ionic strength approaches 0.3 (at pH 7.4), there is a sudden rise in solubility which is most marked between ionic strength 0.25 and 0.30.[12] The data given in Table I illustrate

TABLE I.

Solubility of Cow Muscle Globulin in Potassium Phosphate Buffer at pH 7.4 and 0°.

Preparation No.	Ionic strength.	Protein N dissolved per liter.
		mg.
II A	0.01	0.8
II A	0.10	1.8
II B	0.10	0.0
VI	0.16	0.2
II A	0 20	3.2
VI	0.30	149.0
II A	0.50	1275*

It should be noted that the first five values for solubility given in the table are essentially zero, within the limits of accuracy of the method used.

* Saturation had not been reached; the precipitate had all dissolved.

the order of magnitude of these changes. Stress should not be laid on the absolute values; in the lower ranges, the solubility is so low as to be practically zero at all ionic strengths below 0.2; in the range where the solubility is higher, the amount of protein dissolved varies with the quantity of undissolved protein in equilibrium with the solution. The data of Table I, however, give a good indication of the character of the changes involved, and of the range of ionic strength in which the abrupt change from

[12] This ionic strength range is probably very close to that found within the muscle fiber. In muscle, divalent ions are present in much larger relative amounts than in blood; hence, on the assumption of osmotic equilibrium between the two, the muscle fiber must have a markedly higher ionic strength than blood. For blood $\mu = 0.16$. Preliminary calculations for muscle indicate an ionic strength of 0.22 to 0.26, the protein being disregarded. This subject will be discussed more fully in a later communication.

almost complete insolubility to fairly high solubility takes place. This change occurs regularly for cow muscle globulin near ionic strength 0.3.[13]   Howe (22), in extracting muscles with phosphate buffers, has found that solutions of moderate salt concentration extract far more protein than does pure water; maximal extraction, at a pH near 7.0, was obtained at μ 0.525 (0.225 M) (see also Deuticke (12)).   In all probability the proteins extractible with salts but not with distilled water in Howe's experiments include the fraction considered in these studies, probably as their chief constituent.

It can be seen from these data that muscle globulin may be completely separated from the serum globulins by precipitation in dilute salt solutions.   The classical data of Mellanby (24) for the solubility of serum globulin in dilute salt solutions show that a high degree of solubility is attained at an ionic strength of 0.05, and complete solubility at an ionic strength below 0.10, while muscle globulin remains insoluble until a much higher ionic strength is reached.   Three successive precipitations of muscle globulin at an ionic strength of 0.10 should produce almost complete separation from serum globulin.

The very rapid rise in the solubility of muscle globulin with increasing salt concentration stands in marked contrast with the behavior of oxy- and carboxyhemoglobin in salt solutions, which have been carefully studied by Cohn and Prentiss (9) and by A. A. Green (unpublished).   These substances already possess a fairly high solubility even in distilled water, and their solubility may be increased from 3 to 10 times by the addition of neutral salts.   Muscle globulin, on the other hand, is scarcely soluble at all in water or very dilute salt solutions, while its solubility may be increased more than 1000-fold by the addition of neutral salts, in the ionic strength range 0.2 to 0.4.   Cohn and Prentiss (9) have found that oxyhemoglobin behaves in accordance with the equation of Debye and Hückel like a bi-bivalent or a uni-quadrivalent compound.   The behavior of muscle globulin has not yet been evaluated quantitatively in this manner, but its general behavior is certainly that of a compound of a much higher apparent valence type.   In this respect as well as in its acid- and base-combining

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[13] Preliminary experiments on rabbit protein indicate that passage into solution may take place at a lower ionic strength.

properties and its wide precipitation zone (29), its behavior is quite similar to that of edestin (Osborne and Harris (27)).

Precipitation of Muscle Globulin by Concentrated Salt Solutions.

The concentrations of neutral salts required to "salt out" muscle globulin are in general greater than those required to precipitate fibrinogen and slightly less than those necessary to precipitate euglobulin. The influence of temperature on solubility in the salting out range is very marked, and most of the values given are for 0°.

Muscle globulin at pH 7 and 0° is completely precipitated by 4.4 to 4.6 M NaCl.[14] A distinct decrease in solubility is noted in solutions even at 3.8 M. The change of protein solubility with change in ionic strength is therefore much less abrupt than with the phosphates, which are considered below.

$(NH_4)_2SO_4$ cannot be compared with other salts in its precipitating action on muscle globulin if it is added directly to the protein, for it is sufficiently acid to precipitate the globulin by acidification, apart from the "salting out" effect. If ammonium sulfate be brought to pH 7 by addition of concentrated NH_4OH or NaOH, it will then precipitate muscle globulin when the solution is slightly more than one-third saturated with respect to the salt.

The precipitation by phosphate has been studied in more detail. At 0° and pH 7.4, muscle globulin is still highly soluble at ionic strength 3.0. At ionic strength 3.5 the solubility has become very low. At pH 6.8 a corresponding change in solubility takes place between ionic strength 2.5 and 3.0 (Tables II and III).

Here again the absolute values of solubility obtained fluctuate greatly from preparation to preparation, and are markedly altered by the quantity of undissolved protein present in equilibrium with the solution. The scattering of the values obtained is shown in Table II, and is particularly marked when the solubility is low, the experimental difficulties under these circumstances becoming

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[14] The protein studied here was therefore certainly precipitated with the globulin fraction in the recent studies of Ritchie and Hogan (28) who used saturation with sodium chloride (among other methods) to separate albumin from globulin in extracts of rabbit muscle. Their globulin fraction, however, may have contained other protein fractions as well as that considered here.

greater. The range of ionic strength in which these solubility changes occur is, however, very constant from one preparation to another, after sufficient purification (five or six reprecipitations). These data should, therefore, be of use to those who attempt the separation of muscle globulin from other proteins.

TABLE II.

Solubility of Cow Muscle Globulin in Potassium Phosphate at pH 7.4 and 0°.

Preparation No.	Ionic strength.	Protein N dissolved per liter.
		mg.
II A	3.0	762
III A	3.0	822
II A	3.0	822
II B	3.05	698
II A	3.27	239
II B	3.30	204
II A	3.4	52
II B	3.4	32
II B	3.4	39
II A	3.5	21
II B	3.5	8
II B	3.5	16
II A	3.5	54
II B	3.53	32

TABLE III.

Solubility of Cow Muscle Globulin in Potassium Phosphate at pH 6.8 and 0°.

Preparation No.	Ionic strength.	Protein N dissolved per liter.
		mg.
III A	2.6	565
III A	2.6	598
III A	2.8	42
III A	2.8	31
III A	3.0	14
III A	3.2	6

The influence of temperature on solubility in the "salting out" process is very great. Invariably the solubility of muscle globulin in concentrated salt solutions *decreases* greatly with rise of temperature. This effect of temperature was noted as early as

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1888 by Lewith (23) who studied ox serum proteins, and by Hofmeister (20) in his studies of the precipitating action of salts on egg globulin. Chick and Martin (4) later confirmed these observations for serum proteins, on the alkaline side of the isoelectric point,[15] and A. A. Green has recently demonstrated the same effect in quantitative studies on hemoglobin ((8, 17) and unpublished work). In muscle globulin, the effect of temperature is particularly striking. A solution completely precipitated at room temperature by salting out may entirely redissolve in a short time when cooled to 0°. In one experiment, muscle globulin which was soluble in phosphate at pH 7.4 and μ 3.0 to the extent of 800 mg. of protein nitrogen per liter, had no measurable solubility in the same buffer solution at room temperature.

These observations emphasize the importance of careful temperature control in quantitative (or even qualitative) studies of the precipitation of muscle globulin by neutral salts. Some of the variations found in the solubility studies here reported may well be due to small temperature fluctuations during the process of equilibration and centrifugation.

Use might also be made of these temperature effects in the separation and purification of proteins, especially as the temperature coefficient of solubility appears to differ greatly from one protein to another.

It should be clearly understood that this decrease of solubility with temperature is found only in concentrated salt solutions, where "salting out" takes place. In dilute salt solutions, a very slight increase (instead of a large decrease) in solubility is found for muscle globulin with rising temperature.

Effect of pH on Solubility.

In the absence of salt, as Salter (29) has reported, muscle globulin is insoluble over a wide pH range, roughly 4.5 to 8. In the presence of moderate salt concentrations (above ionic strength 0.3 and below the salting out range) it is readily soluble if the pH is maintained alkaline to 6.0. There is a narrow zone around pH 6.0, on the acid side of which the protein becomes insoluble even

[15] They found that on the acid side above 9° rise of temperature has the opposite effect.

in the presence of salt concentrations which would readily dissolve it at a slightly more alkaline reaction.   If the pH is brought back to about 7, the protein redissolves if sufficient salt is present. There is generally, however, some insoluble residue which dissolves only on the addition of a considerably larger amount of alkali.   The amount of insoluble protein left becomes larger, the longer the protein has been left in the precipitated condition. Weber ((32) p. 487) also noted that myosin precipitated by acid was only partially redissolved at pH 8.   This alteration into an insoluble form is notably similar to the conversion of edestin into edestan (Osborne (26)).

On the addition of further acid to the precipitated protein, it redissolves (at about pH 4.7) and forms a clear solution (the "acid albumin" or "syntonin" of earlier observers), which is not precipitated (although altered in its properties) by the addition of further acid.

If the solution is alkaline to pH 6.0, the addition of further alkali does not precipitate the protein, but it is markedly altered by reactions alkaline to pH 10.5.[16]

Viscosity and Hydration.

The viscosity of muscle globulin is of a higher order of magnitude than that of the blood proteins or of myogen.   A solution containing 1 gm. of muscle globulin per 100 cc. flows about 10 times as slowly through an Ostwald viscosimeter as the salt solution in which it is dissolved, while any of the blood proteins at this concentration is only slightly more viscous than pure water.

Dr. Jeffries Wyman, Jr., in the course of investigations in this laboratory on other proteins, has made a number of determinations of the apparent[17] viscosity of muscle globulin, some of which are reproduced in Fig. 1.   Weber's (34) values for myogen (pH 1.46) and Chick's (2) values for euglobulin (pH 5.5 to 6.0) and serum albumin, are given for comparison (the values from Chick's data are interpolations, as all her measurements were made on higher protein concentrations).

[16] The alteration of the protein by acid and alkali is discussed in the following papers by von Muralt and Edsall.

[17] Reasons for regarding these viscosity values as only "apparent" are given in the following paper by von Muralt and Edsall.

All the measurements given were made with Ostwald viscosimeters. Weber's measurements were made at 19°, all the others at 25°. The values for η_0 in each instance are for the particular solution in which each protein was dissolved (salt solution or pure water).

A solution containing 1.5 per cent of muscle globulin is a thick syrup which crawls rather than flows. If the protein solution is
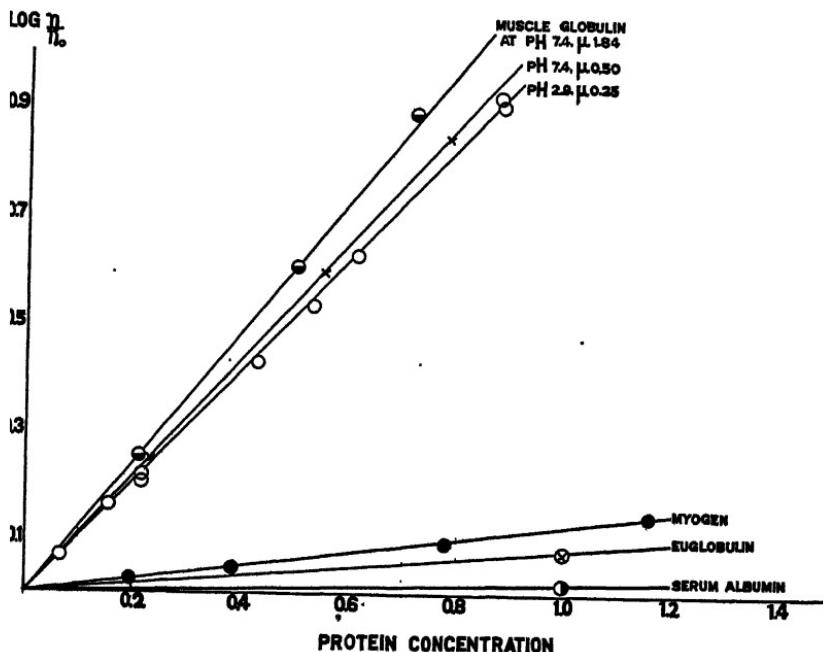
Fig. 1. The concentration of protein, measured in gm. per 100 cc., plotted against the logarithm of relative viscosity.

concentrated further by being placed in a vacuum desiccator with phosphorus pentoxide, it generally sets to a gel.

It should be noted that the precipitated protein, even after prolonged centrifugation, remains as a jelly-like mass containing 98 to 99 per cent of water. Attempts to remove this water and dry the protein have invariably failed, unless the protein was denatured in the process. Even the denatured protein tends to precipitate in the form of a thick spongy jelly containing a large amount of water, which can be removed only by vigorous grinding,

treatment with alcohol and anhydrous ether, and prolonged heating in an oven at 100–110°. The characteristic salt-soluble protein seems to possess an extraordinary affinity for water, and cannot be separated from water without some radical change in the protein itself. This property may well be of physiological significance. It is interesting to note for instance, that Gamble (16) and his coworkers have noted no significant change in the water content of skeletal muscle, even in cases of profound dehydration, when the fluid of the intercellular spaces was profoundly depleted, and the blood volume was diminished well below normal. If loss of weight in the muscles did occur, proteins and salts were lost to the muscle along with the water removed, so that the chemical constitution of the muscle remained essentially constant even while its weight diminished. In other words, the muscle appears to lose water to any extreme degree only when the protein present is broken up and lost as well. The behavior of muscle globulin suggests that the water is directly held by the protein, and that this water-holding power is not lost when the protein is extracted from the muscle.

Gel Formation.

Salter (29) notes: "the addition of alkali to a suspension of isoelectric protein renders it at first extremely viscous, but finally completely dissolves the protein." This point is sufficiently interesting to warrant further discussion. The above phenomenon occurs only in the almost complete absence of salt. The isoelectric precipitate under these conditions is a dense opaque jelly-like mass. On the addition of very small amounts of alkali (enough to bring it into the pH range 6.5 to 8.5) the rigidity of the gel structure increases markedly, while at the same time the whole mass becomes almost completely transparent. On further addition of alkali, the rigidity of the gel diminishes, and in the neighborhood of pH 10 the gel structure apparently disappears, and a clear solution results.

Gels can also be formed, as described above, by evaporating down an already concentrated solution of muscle globulin; but the gels here described are peculiar in that they remain firm and rigid even when the protein concentration is extremely low. Charac-

teristic gels have been obtained, containing only 0.2 per cent of protein by weight.

As stated above, the salt concentration must be low if gels of this type are to be formed. In cow muscle globulin, the salt concentration must apparently be reduced to 0.001 N, or below; in rabbit muscle globulin, the formation of a gel can occur when the salt concentration is a little below 0.01 N. The addition of a small amount of salt to a gel produces rapid disappearance of the gel structure, a fine flocculent precipitate of protein being formed instead. On the addition of more salt, the precipitate dissolves, and the characteristic phenomena of solution by neutral salts are observed. The behavior of these gels is further discussed in the following paper by von Muralt and Edsall.

Relation of Muscle Globulin to Proteins Studied by Other Investigators.

Beginning with the work of Kühne (1868) muscle proteins have been studied by a large number of investigators who applied a varied terminology to the substances they discovered. A word as to the relation of this work to that of other investigators is therefore in place.

The myosin of Danilewsky (11), prepared by extracting muscles with 10 to 15 per cent ammonium chloride, and subsequently precipitating the protein by dilution of the extract with water, is in all probability identical with the protein here considered. The protein to which von Fürth (14, 15) applied the name myosin appears to be the same; it should be noted that von Fürth extracted rabbit muscles with 0.6 per cent sodium chloride, probably an inadequate solvent for muscle globulin; the quantities of myosin that he obtained were therefore, as he notes, small. (See the discussion of this point by Howe (22) and note Howe's figures for the extraction of muscle proteins by salts of varying concentration.) Von Fürth, however, noted carefully the quantities of neutral salts necessary to salt out myosin, the precipitation of the protein by dialysis and by small quantities of acetic or mineral acids, and its solubility in excess of acid. In all these respects its behavior corresponds with that of the muscle globulin considered here. It seems also justifiable to identify it (as does von Fürth) with Halliburton's (18) paramyosinogen. Halliburton, to be sure,

found no precipitation on the addition of acetic acid to a solution of the protein; but it seems justifiable to accept von Fürth's explanation, that Halliburton had added an excess of acid, in which the protein is soluble.

The myosin of Weber (32) is also in all probability to be identified with the protein here considered. The only seeming discrepancy between the two arises in regard to the apparent isoelectric point[18] (see above). Muscle globulin certainly cannot be confused with myogen. Myogen is soluble in pure water to the extent of at least 5 to 6 per cent; and this fact at once excludes any possibility that the two proteins might be identical. The extraordinary difference in their viscosity (see Fig. 1) should also be noted.

The protein studied here must also be, at least in part, identical with that fraction in Howe's (22) studies, which was extracted from muscle by 0.225 and 0.525 M phosphate, but not by water nor by 1.425 M phosphate. It is also included with the globulin fraction of Ritchie and Hogan (28), but their globulin fraction may well have included other proteins besides that considered here.

Our muscle globulin is distinct from the "soluble myogen fibrin" of von Fürth. The latter is apparently an alteration product of myogen, which behaves like a globulin in being precipitated by dialysis. It coagulates rapidly at 30–40° and slowly in the cold. It forms in muscle extracts, especially in the presence of salt and probably in unbuffered solutions which are gradually becoming more acid. (See Howe's (22) discussion of this point,

[18] Dr. Weber, in personal correspondence, has very kindly furnished us with many data regarding the behavior of myosin, which he has obtained in extensive investigations not yet published. These data have been of the greatest value in clearing up doubtful points and make it almost certain that muscle globulin and myosin are identical. Weber's experimental data have not been found to conflict with ours on any point. Several interpretations are possible of the experiments on isoelectric point, and on acid- and base-binding, but the substance under study in the two laboratories appears to be identical.

The name muscle globulin is retained in this paper, and in two following papers by von Muralt and Edsall, to describe the protein preparation here studied. Reasons are given by von Muralt and Edsall for giving the name myosin to one specific constituent of the still impure muscle globulin preparation.

and Weber's (32) data on the precipitation of myogen in slightly acid solutions by various anions.) Its concentration therefore, increases steadily as the extract is allowed to stand. In these studies on muscle globulin, on the other hand, a heavy precipitate was always obtained on diluting the original extract with water, even 4 or 5 hours after the animal was killed, the whole preparation having been carried out in the cold. The amount of precipitate did not noticably increase if the dilution took place 24 hours or more after extraction. Muscle globulin appears therefore to be a substance originally present in the muscle extract, not an alteration product of a water-soluble protein. From von Fürth's discussion it is not altogether clear whether soluble myogen fibrin can be repeatedly reprecipitated and redissolved by neutral salts, but apparently it cannot, as it soon passes over into a permanently insoluble form, myogen fibrin. In this respect again, the behavior of muscle globulin is entirely different. It can be precipitated again and again by increasing or decreasing the ionic strength, and always readily redissolved (if the necessary precautions are observed). Kept dissolved in buffered neutral salt solution in the cold, muscle globulin remains soluble for months, only very small traces of insoluble materials being formed in this time. In all these respects the behavior of muscle globulin would appear to be distinctly different from that of soluble myogen fibrin.

Bottazzi and Quagliariello (1) have observed fresh muscle juice to be filled with enormous numbers of minute granules, invisible to the most powerful microscope, but visible in the ultramicroscope. These granules readily agglutinated, under the influence of heat, or of the addition of water and weak acids (such as Na-H_2PO_4), to form visible precipitates. Agglutination also occurred spontaneously on standing. The precipitate so formed was not dissolved by acids or alkalies, and the particles were not dissolved by neutral salts. Bottazzi and Quagliariello believed these particles to be identical with the myosin of von Fürth.

There are significant differences, however, between the ultramicroscopic granules and the protein considered in this paper. Solutions of muscle globulin show an empty field when examined with the ultramicroscope. Occasionally a few large, coarse particles (without Brownian movement) are visible; there is

nothing which in the least suggests the innumerable brilliant granules described by Bottazzi and Quagliariello. The physico-chemical behavior of muscle globulin is also very different from that of the granules. The latter are insoluble in neutral salt, and in weak acids and alkalis, whereas muscle globulin is readily soluble in both salts and weak alkalis, and in a moderate excess even of weak acids. Nor does muscle globulin precipitate spontaneously, except slowly and to a very slight extent, on standing.

It should be remembered in this connection that expressed muscle juice is a fluid of weakly acid reaction. Weber (32) records that the pH of the muscle juice which he obtained ranged from 6.3 to 5.8. Muscle globulin becomes insoluble even in salt solutions at a pH close to 6.0. It may well be that Bottazzi and Quagliariello were observing very small aggregates of muscle globulin molecules which had formed in the presence of the weakly acid medium. With other proteins (egg and serum albumin, etc.) Chick and Martin (3) observed that near the isoelectric point (but not so near that complete agglutination occurs) protein particles are visible in the ultramicroscope, even though entirely invisible to the naked eye. The same may well be true of the muscle proteins. The hypothesis that the granules are formed by the action of acid on the muscle proteins was indeed discussed and rejected by Bottazzi and Quagliariello, but their grounds for rejection should perhaps be reexamined in the light of physicochemical data which have been accumulated since their work appeared.

Another hypothesis suggests itself: that instead of agglutination products, the ultramicroscopic granules may be pieces of preformed structural units in the muscle fiber (made up, perhaps, largely of myosin) which have not been broken up enough to pass into molecular solution. The methods of preparation and purification described in this paper would probably eliminate the granules, if such were their nature, and keep only the myosin which had passed into solution. Muscle globulin in fact may bear an extremely close relation to the granules studied by Bottazzi and Quagliariello, but the two are clearly not identical.

SUMMARY.

1. Further data concerning the muscle globulin of the cow, previously studied in this laboratory by Salter and Heyroth, are

presented.   The studies have also been extended to rabbit muscle globulin.

2. The globulin is prepared by extraction of finely chopped fresh muscle with salt solutions (KCl and potassium phosphate) of ionic strength 1.2 to 1.5 and pH 7 to 8.5.   The protein is precipitated by increasing or decreasing the ionic strength of the filtered extract.   It may readily be redissolved and reprecipitated many times without any apparent change in its properties. If kept in the cold, protected from bacteria, dissolved in salt, at pH 6.5 to 7.5, its properties appear unchanged over a period of several months.

3. Weber has found the isoelectric point of the salt-free precipitated protein to be at pH 5.1 to 5.5.   Salter and Edsall find a minimum of acid- and base-binding capacity at pH 6.2 to 6.6.

4. Muscle globulin remains insoluble at ionic strengths which completely suffice to dissolve serum globulin.   At pH 7.4 in phosphate it first becomes appreciably soluble between ionic strength 0.25 and 0.30.   In the range, 0.2 to 0.4 in ionic strength (a range which includes the ionic strength obtaining in the muscle), the solubility increases more than a 1000-fold.   The solubility values fluctuate from one preparation to another, and are markedly affected by the amount of undissolved protein in equilibrium with the solution.

Muscle globulin precipitates at high ionic strengths less readily than fibrinogen, but somewhat more readily than euglobulin. Data describing its precipitation by neutral salts are given.   Temperature has a great influence on the salting out process, rise of temperature causing a great *decrease* in the solubility of the protein.

The protein is insoluble at all salt concentrations between pH 5 and 6.   As the acidity becomes greater than pH 5, the protein redissolves and in this range does not require the presence of neutral salt to dissolve it.   Alkaline to pH 10, it is also soluble in the absence of salt.

5. Its apparent viscosity is of a higher order of magnitude than that of the serum proteins or of myogen.   Even concentrated precipitates of the protein contain 98 per cent of water, and it can apparently remain undenatured only in the presence of a large amount of water.   Possible physiological bearings of this fact are discussed.

6. In the nearly complete absence of salt, and in the presence of small amounts of alkali, gels of the protein are formed, of a peculiar character.

7. The muscle globulin here studied appears to be identical with the myosin of Danilewsky, von Fürth, and Weber, and the paramyosinogen of Halliburton. It also appears to correspond largely with the fraction which in Howe's studies was extracted from muscle by moderate salt concentrations, but not by distilled water or by high salt concentrations. It is not von Fürth's "soluble myogen fibrin." Its relation to the ultramicroscopic granules of muscle juice, studied by Bottazzi and Quagliariello, is uncertain. It may be closely related to these granules, but cannot be identical, for muscle globulin solutions show no particles in the ultramicroscope.

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STUDIES IN THE PHYSICAL CHEMISTRY OF MUSCLE GLOBULIN.

III. THE ANISOTROPY OF MYOSIN AND THE ANGLE OF ISOCLINE.

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The muscle globulin preparation, which has been studied in recent years in this laboratory successively by Salter (29), Heyroth,[1] and Edsall (8) has been found to produce double refraction of flow. A brief report on this effect has previously been published (23). The investigation has since been carried further and yielded quantitative data, which tend to confirm the view, that in muscle globulin there is a highly anisotropic protein, which is the cause of the double refraction in the intact living muscle fiber. The study of this anisotropic protein fraction by the method of double refraction of flow is complicated, two independent effects being involved. The double refraction which appears while a muscle globulin solution is flowing is primarily due to orientation of anisotropic particles. This effect, however, is always accompanied by a certain amount of double refraction which is produced by the elastic deformation of these particles, due to the shearing stresses which occur in flow. To simplify the presentation of the material the two phenomena are separately discussed, although the actual measurements of the two effects were made simultaneously. In this paper the relation between the orientation and elastic deformation of the particles is discussed on the basis of measurements of the "angle of isocline" (Kreuzwinkel of Zocher (37)). The following paper on double refraction of flow (Strömungsdoppelbrechung) deals with the anisotropy of the streaming particle.

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[1] Unpublished experiments in the files of the Department of Physical Chemistry.

Method.—The muscle globulin preparation, which has been described from the chemical point of view in the preceding paper (8) is a colorless, viscous fluid with great light-scattering power. Under the ultramicroscope, however, as well as under the polarizing microscope, the fluid appears optically void. If muscle globulin is allowed to flow through a fine capillary, or if the solution is subjected to shearing stresses between two concentric cylinders, it shows a considerable amount of double refraction. This effect can be observed qualitatively very easily by an arrangement similar to that described by Zocher (37). The muscle globulin is placed in a beaker and stirred either by a glass rod or by simple rotation. If the solution is observed through a nicol in plane-polarized light, which can be conveniently produced by a set of glass plates, it appears dark, provided the planes of polarization of the incident light and the nicol are crossed. As soon as the fluid is set into motion, the field of vision becomes bright, the plane-polarized light being changed into elliptically polarized light which cannot be extinguished by the observing nicol. The sign of the double refraction is positive with respect to the direction of flow.

This effect is known as double refraction of flow (fluxional birefringence, Strömungsanisotropie of Freundlich). Various colloids (3, 4, 14, 37, 38) have been found to give double refraction of flow. The classical example of this phenomenon is now the V_2O_5 sol, so extensively studied by Freundlich, Stapelfeld, and Zocher (15). For this reason the double refraction of muscle globulin will be compared frequently in these papers with the behavior of the V_2O_5 sol which has been so admirably described in quantitative terms. Not only colloids, but also pure liquids may give double refraction of flow, as Vorländer and Walter (35) have shown with 172 pure liquids. Vorländer and Walter, however, implied that the type of double refraction which they obtained (on higher alcohols for instance) was sharply distinct from the double refraction of flow in colloids. Since then it has become more and more apparent through the development of the theory of molecular double refraction of flow by Raman and Krishnan (26) that molecular and colloidal double refraction of flow are much more closely related than Vorländer and Walter were inclined to believe. The relations between these two classes of substances with respect to

double refraction will be discussed in connection with the results on muscle globulin, since it appears to be an interesting intermediate substance. Among the proteins hitherto only gelatin solutions have been found to show double refraction of flow (1, 5).

Two different types of apparatus have been employed for investigations on double refraction of flow. The principle of both forms is to produce orientation in the liquid to be tested by means of shearing forces. If the liquid is doubly refractive plane-polarized light is transformed into elliptically polarized light in passing through it. The phase difference of the two components of the elliptically polarized light is functionally related to the orienting force in the liquid and can be measured with the aid of a compensator. The original form of the apparatus producing flow is that consisting of concentric cylinders, devised by Kundt (19), and more recently used by Vorländer and Walter (35). A modification of this apparatus has been adapted for the experiments on muscle globulin. The fluid is plated in the annular gap between two concentric cylinders, one of which rotates while the other is at rest. The layer close to the rotating cylinder moves with the same velocity as the cylinder; the layer close to the stationary cylinder is assumed to be at rest; and the intermediate layers move with increasing velocities the closer they are to the rotating cylinder. Each concentric layer of the fluid moves with a different velocity and tends to retard or accelerate adjoining layers. Between the adjoining layers shearing forces therefore arise which are largely responsible for the double refraction of flow. Kundt as well as Vorländer and Walter had to employ in their apparatus very high velocities of revolution in order to obtain small amounts of double refraction in the fluids which they studied. Muscle globulin, however, exhibits a great amount of double refraction in the concentric cylinder apparatus with velocities as low as 30 R.P.M., and in this respect bears a much closer resemblance to the V_2O_5 sol.

Another type of apparatus was introduced by Diesselhorst, Freundlich, and Leonhardt (7). In this arrangement the shearing forces are produced by flow through an oblong cell with rectangular cross-section. In this case the fluid layer next to the walls of the cell remains at rest, while at the center the fluid flows out with maximum velocity. If the cell through which a doubly refractive

fluid is flowing is viewed in polarized light at right angles to the direction of outflow, double refraction occurs as if the cell were a uniaxial birefringent crystal whose optic axis lies (in most cases) in the direction of flow.

There is an important difference between this type of apparatus and that composed of concentric cylinders. In the latter, since the direction of observation is parallel to the axis of rotation, each pencil of light which passes the annular gap traverses a concentric layer of constant velocity. In the outflow cell, the direction of observation is perpendicular to the direction of flow and therefore each pencil of light on its way from one of the walls to the other passes through layers of varying velocity. This results because the layers in the center of the cell are moving with maximum velocity and those close to the walls are stationary. The shearing forces which are exerted vary with the position of the layer in respect to the cell wall. The shearing force for fluids with normal viscosity can be calculated for each layer; and can be shown to decrease as a linear function of the distance from the cell wall, becoming 0 at the center of the cell. In a liquid like the V_2O_5 sol or muscle globulin these relations become more complicated, and the distribution of the shearing forces in the outflow cell cannot at present be calculated. In the concentric cylinder apparatus on the other hand each pencil of light has passed through a layer of constant velocity, which has been subjected therefore to a constant shearing force. In so far as the experimental determination of the relation between shearing force and double refraction was one of the aims of this investigation, the outflow cell seemed unsuitable and an apparatus composed of concentric cylinders was constructed.

If a doubly refractive fluid is placed between concentric cylinders, the particles at rest are maintained in completely random orientation because of their temperature motion. In Fig. 1, a each particle is schematically represented by a line indicating its optic axis. If a fluid shows double refraction of flow, it is a necessary condition that the molecules or particles be asymmetrical with respect to shape, as well as anisotropic with respect to optical properties (26). The optic axis of the particle, however, does not necessarily coincide with the longest axis of geometrical asymmetry. This coincidence may occur, but cannot be assumed without special proof. It should be emphasized therefore that

the lines in Fig. 1 represent the optic axis of the particles, and not necessarily the largest axis of geometrical asymmetry.

When the external cylinder is set into motion laminar flow occurs in the liquid between the two cylinders, and shearing forces arise between the adjoining concentric layers in the fluid. These forces produce a definite orientation of the asymmetric particles, as well as a certain amount of elastic deformation. Thus results a situation similar to that represented in Fig. 1,b. Orientation of the geometrically asymmetric particles orients also their optic axes, which now act additively as if the annular space were one large
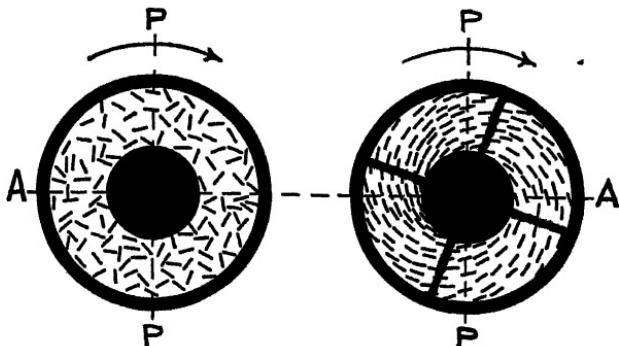
FIG. 1, a.   FIG. 1, b.

FIG. 1. Orientation of particles in a doubly refractive fluid placed between concentric cylinders; Fig. 1, a, particles, each schematically represented by a line indicating its optic axis, at rest; Fig. 1, b, orientation due to motion of external cylinder.

spherocrystal. Accordingly a black cross appears if the annular space is viewed in plane-polarized light through a nicol in a direction parallel to the axis of the concentric cylinders. The arms of the cross represent those azimuths in which the oriented optic axes of the particles coincide with one of the planes of polarization, since for such azimuths the plane-polarized light passes through unaltered and is therefore extinguished by the analyzer. This cross is here called the "cross of isocline" as a translation of the term "Wirbelkreuz." The smaller of the two angles which the optic axis of the oriented particle forms with the radius drawn to this particle is called by definition the *angle of isocline*, Ψ, in accordance with the identical term "Kreuzwinkel" introduced by Zocher

(37). The angle of isocline, Ψ, is at the same time the smaller of the two angles between the optic axis of the particle and the direction of the velocity gradient between two adjoining layers, as well as the larger of the two angles which the cross of isocline forms with the planes of polarization, AA and PP. The black cross of isocline provides, therefore, the experimental means to measure the angle of isocline, Ψ.

Theoretically angles of isocline, Ψ, between 45° and 90° might be expected. In Fig. 2 three different crosses of isocline are drawn, such as appear in a very old V_2O_5 sol (Fig. 2, a), in a young V_2O_5 sol (Fig. 2, b), and in muscle globulin (Fig. 2, c).

The angle $\Psi = 90°$ (Fig. 2, a) indicates that the optic axis of the particle lies parallel to the stream-lines. From the hydrodynami-
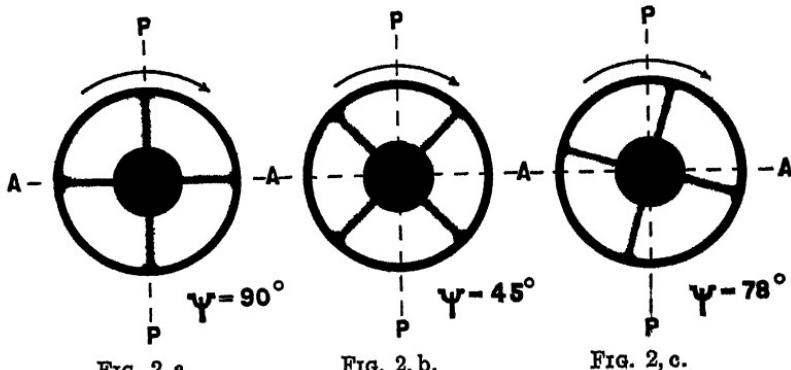
FIG. 2, a.   FIG. 2, b.   FIG. 2, c.

FIG. 2. Crosses of isocline as they appear in very old V_2O_5 sol (Fig. 2, a), in young V_2O_5 sol (Fig. 2, b), and in muscle globulin (Fig. 2, c).

cal point of view such an effect is to be expected if it can be assumed that the optic axis is also the longest axis of the asymmetrical particle. In this case the particle obtains its orientation in the direction of the stream-lines, because in any other position it will lie with its ends in layers of different velocities, which exert an orienting couple upon it. If double refraction of flow were purely a matter of orientation of anisotropic, asymmetrical particles, angles of isocline of 90° would be the rule, provided that the optic axis coincides with the direction of largest asymmetry. Deviations from the 90° angle could be explained by a deviation between the optic and the geometrical axis, a phenomenon well known in

mineralogy. But this simple explanation does not hold. The needle-shaped V_2O_5 particles for instance may give angles of isocline between 45° (Fig. 2,b) and 90° according to the age of the preparation. From other data it is known that in the V_2O_5 particle the optic axis and the long axis of the needle coincide. The angle of isocline gives therefore a true picture of the orientation of the needles. The validity of this simple picture of orientation being assumed, an angle $\Psi = 45°$ (Fig. 2,b) would be called anomalous. Curiously enough, the earlier investigators in the
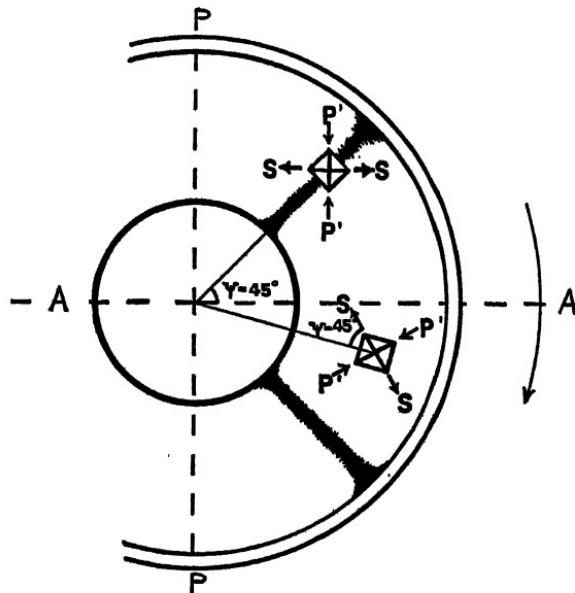
Fig. 3. The shearing forces of an element of a fluid. SS represents stress, $P'P'$ represents pressure (after Stokes).

field, Maxwell, Kundt, de Metz (22), and their pupils (34), were troubled by the opposite problem. From their point of view $\Psi = 45°$ was normal and deviations from such an angle were described as anomalous. These investigators based their views upon Stokes' (31) theory of a fluid flowing between two concentric cylinders. Stokes showed theoretically that the shearing forces on an element of the fluid could be represented by stress in the direction SS (Fig. 3) and pressure in the direction $P'P'$. If the fluid could be considered as a homogenous, elastic substance,

double refraction may be expected as a consequence of the elastic deformation produced by SS and $P'P'$.

The optic axis of this accidental double refraction, produced by stress and pressure, lies in the direction SS, since $P'P'$ and SS taken separately would produce the same effect. The angle of isocline therefore would be $\Psi = 45°$. The double refraction produced by elastic deformation of an isotropic substance is called photoelastic effect. The most commonly quoted example of this type of accidental double refraction is the photoelastic effect in glass under stress or pressure. Glass however is an unfortunate example, since it has been shown that in most so called photoelastic substances orientation of anisotropic crystalline micelles is largely responsible. A pure photoelastic effect is best demonstrated in the crystals of the cubic system, where the possibility of such orientation effects is excluded. In these crystals there is no doubt that elastic deformation of the regular crystal lattice is alone responsible for the double refraction. Bragg (6) and Ewald (10) have assumed that the anisotropic Lorentz-Lorenz forces, due to the anisotropic arrangement of ions in the strained condition, could account for the photoelastic effect and have based their calculations on this assumption. Herzfeld (17), however, has recently shown that even for such a comparatively simple system as the cubic crystal, this assumption is insufficient to account for the experimental data. From this it is apparent that what is called accidental double refraction under tension or pressure in gels and other colloidal systems must be a complicated effect involving both orientation of anisotropic crystalline micelles and the photoelastic effect; the latter being in itself a complicated phenomenon. (An excellent review of accidental double refraction in colloids has been given by Wächtler (36) and also in Ambronn and Frey's book (1). Kunitz (20) has very recently employed the photoelastic effect in gelatin for the determination of the internal stresses during swelling.)

All of the early investigations on double refraction of flow were undertaken with the aim of discovering the photoelastic effect in fluids. From this point of view, therefore, angles of isocline other than 45° were anomalous. When double refraction of flow in colloids was discovered and the hypothesis of orientation of anisotropic micelles rapidly gained experimental support, these early

investigations all fell into discredit, especially since the early investigators had unknowingly employed a number of colloids for their research. It was thought that double refraction of flow was merely an orientation effect of colloidal micelles, and that photo-elasticity in the strict sense of the word did not exist in fluids. This view was very clearly expressed by Björnståhl (5) who thought that only colloids showed double refraction of flow. At present this conception cannot be maintained, since Vorländer and Walter (35) have found the effect in pure organic compounds. A discussion of the views which we have formed with respect to the interpretation of the angle of isocline will be given together with the discussion of the results with muscle globulin.

Apparatus.—The apparatus, which was designed and built for the investigation of the double refraction of flow of muscle globulin, consists of rotating concentric cylinders, thermostat, and optical bench, with light source, lenses, nicols, and compensator mounted on special riders.

Arrangement of Concentric Cylinders (Fig. 4).—The concentric cylinders are mounted on a stand which consists of two heavy cast-iron rings (R) connected together by three steel bars, the lower acting as base, the upper carrying the ball bearing with the moving parts. The ball bearing is of S.K.F.[2] manufacture, 6 cm. inside diameter, insuring quiet and steady rotation of the inner parts up to velocities of 1600 R.P.M. if necessary. The brass mounting (M) fits into the inner ring of the bearing held thus by the shoulder on the lower rim and by four screws, which connect the chromium-plated disc (P) with the mounting. M is at the same time pulley for the driving belt. The disc (P) protects the ball bearing against dust and chemicals and carries the revolution counter (C) which is a little flat chromium-plated spiral, mounted in a groove. This spiral, acting as an endless screw, advances the notches of the small gear (g) with each revolution. Multiplying the revolutions of the disc by 38, the number of notches on the gear, gives the number of revolutions, and therefore the velocity of the apparatus. For most of the measurements, however, the device was used as an indicator of one completed revolu-

[2] Svenska Kulelager Fabrik.

tion of the disc, as it gave a little click. The clicks could be conveniently counted with a stop-watch up to 360 R.P.M.

It is most important that the inside of the glass cylinder (G) should be true with respect to the axis of revolution. It is practi-
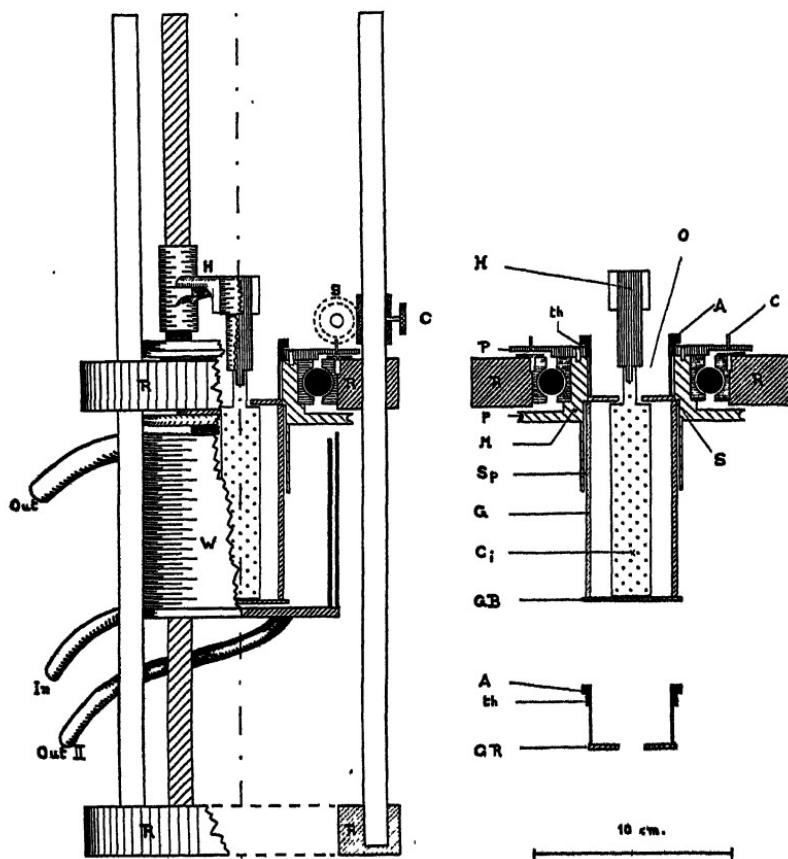
FIG. 4. Apparatus designed for the investigation of the double refraction of flow of muscle globulin showing the arrangement of the concentric cylinders.

cally impossible to find glass tubing of 4 cm. inside diameter, which meets this requirement. It was therefore necessary to grind these glass cylinders specially on the inside, in order to make them truly round. The glass cylinders we have used were all ground on the inside, and revolved true with respect to the axis of the

apparatus. One disadvantage which could not be overcome was that, because of the grinding, they were slightly conical, having the smallest diameter in the middle and the largest on both ends. This however in no way influenced the measurements. The attachment of the glass cylinder to its brass mounting required many unsuccessful experiments. The following procedure, although not entirely satisfactory, proved best. For each glass cylinder a special brass holder was made (the inside diameters varied slightly as a result of the grinding), fitting closely into the inside of the glass cylinder and also tightly into the round opening (O) of the mounting (M). This holder was inserted into the mounting and the glass cylinder was pushed over it from the other side until it touched the shoulder (S). In this position the glass cylinder was concentric with the holder and the mounting with respect to its inside diameter. Between the mounting (M) and the glass cylinder is a small annular space (Sp), which then was filled up with paraffin.

Metal and glass are not readily sealed together with paraffin. The glass cylinders were therefore first wrapped with adhesive plaster and then sealed with paraffin. This proved to be a secure method of fixing them to their metal mountings. Previous to being sealed to the mountings, the glass cylinders were provided with a bottom of plane glass (GB) which was sealed on, either with De Khotinsky cement, or picein. As soon as the paraffin was hard, the brass holder on the inside was removed, and the cylinder was ready for use. The inner cylinder (Ci), which could be moved up and down on its holder (H) in a dove-tailed slider on one of the steel rods was in the later experiments a chromium-plated copper cylinder. In the earlier experiments a glass cylinder was used, but great difficulties in centering were experienced. Later, unsatisfactory results were obtained with a platinum-plated metal cylinder. The platinum plating did not stand the combined corrosive effects of repeated cleaning and the sulfur in the protein solutions. The chromium plating however proved to be completely resistant towards the protein, was very easily cleansed on account of its brilliant surface, and the proteins, unless left in contact with it a long time, showed no signs of denaturation.

The dimensions of the cylinders used were: Cylinder 1, radius $r = 0.91$ cm., length $l = 9.9$ cm.; Cylinder 2, $r = 1.30$ cm., $l =$

9.9 cm.; Cylinder 3, r = 1.80 cm., l = 9.9 cm.   Accordingly the volume of the annular space varied.   With Cylinder 1 the volume V_1 was 105 cc.; Cylinder 2, V_2 = 80 cc.; Cylinder 3, V_3 = 55 cc.

The apparatus is filled with about 10 cc. more than the volume required to fill the annular space.   Then the inner cylinder (Ci) is lowered into the solution until it almost touches the glass bottom of the external cylinder.   The muscle globulin solution at this stage stands higher in the cylinder than in Fig. 4.   The annular brass carrier (A) for the glass ring (GR) is next lowered through the opening (O) until the glass touches the meniscus of the muscle globulin solution.   Care is taken to eliminate all air bubbles and then the brass carrier (A) is screwed in by the thread (th) which screws into the disc (P) until the glass ring (GR) touches the upper rim of the outer glass cylinder (G).   The superfluous 10 cc. of solution can now be sucked out with a pipette and the upper surface of GR dried and cleansed, thus insuring an undisturbed passage of the light rays through a plane parallel layer of solution between GR and GB.

The length of the layer through which the light passes is of course equal to the length of the cylinder G and was in all experiments 10 cm.   The protein solution is thus in contact with glass on three sides and with the chromium-plated inner cylinder (Ci).

Thermostat and Rotating Arrangement.—The concentric cylinders are kept at a low temperature in order to protect the protein solution from denaturation during long experiments, and in order to maintain constant shearing forces which among other factors are a function of viscosity.   This is accomplished by constantly pumping water through the water bath (W) from a large thermostat. The water is pumped from the bath by a Ford motor car pump which runs at about 2000 R.P.M., through the inlet (In, Fig. 4) and runs back to the thermostat by gravity through the outlet (Out).   The flow is regulated by a water faucet (not visible in Fig. 4) and to protect from unexpected overflow the water bath (W) is built in the form of two concentric brass cylinders, with a glass bottom sealed on with De Khotinsky cement.   The inlet opens into the inner cylinder, which is slightly shorter than the outer cylinder.   The outlet which has twice the diameter of the inlet takes water out near the upper rim of the inner cylinder.   In case the outlet cannot dispose of all the water, it passes over

the rim of the inner cylinder, flows down into the annular space, and runs out by the safety outlet (*Out II*) which empties the annular space at the bottom. This device functioned efficiently for more than a year and could be kept running any desired length of time without attendance.

The glass cylinder (*G*) in the brass mounting (*M*) is driven by a small elastic leather belt acting on the pulley (*p*) connected with the mounting (*M*). The ball bearing (*B*), as well as the elastic belt, insures a steady rotation. The leather belt runs from the pulley (*p*) to a double set of reducing pulleys located about 10 cm. from the concentric cylinders. With these pulleys the velocity of rotation can be changed from 30 up to 360 R.P.M. while the $\frac{1}{6}$ horse-power General Electric motor runs practically at full speed. The motor with the pulleys is conveniently mounted at the height of the pulley (*p*) on the wooden bench (*B*) shown in part in Fig. 5.

Optical Bench, Fig. 5.—All the optical parts are mounted separately in special riders on a large universal optical bench, manufactured by E. Leitz, Wetzlar, on which they slide up and down the cast-iron rails (*R*). These rails can be rotated around a vertical axis of about 90°, or around a horizontal axis through the same angle. Furthermore they can be moved up and down to any position in the large cast-iron stand (*St*). All manipulations are very smooth, since the movable parts are counterbalanced by a large weight (not visible in Fig. 5). As light source an automatic feed arc lamp (*A*) of 5 to 8 amperes is mounted on the extreme end of the bench, the carbons of which are regulated by clockwork. The lamp as a whole can be centered. Close to the lamp is the first condenser (*C_1*) which has a high relative intensity and which stands the heat of the arc at a distance of 3 cm. Below this condenser the polarizing nicol (*PN*) is mounted so that the light passes through in focus. This nicol prism has a special outlet for the extraordinary beam, which prevents it from overheating. The condenser C_2 gathers the strong beam of divergent plane-polarized light into a practically parallel beam. This beam passes through the plane parallel layer of muscle globulin solution between the two concentric cylinders, *Ci* and *G*, enclosed by *GR* and *GB*. The saddle stand on which the condenser C_3, the half-shadow wedge (*HS*), and the quarter wave-length plate $\left(\frac{\lambda}{4}\right)$ are mounted, has a
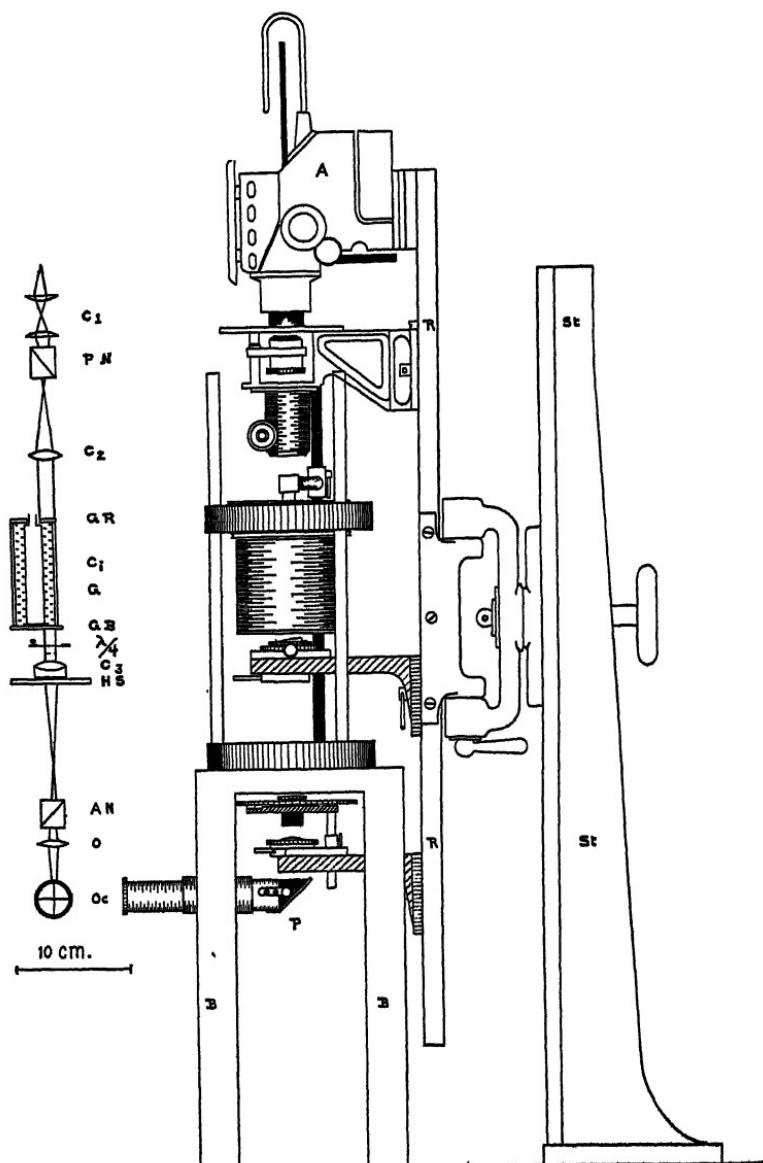
FIG. 5. Optical bench of the apparatus designed for investigation of the double refraction of flow of muscle globulin.

device by which these attachments can independently be taken out of the optical path. In the experiments for determination of the angle of isocline, these devices were only used for adjusting and centering purposes. Since they form an essential part in the investigation on double refraction of flow, a detailed description will be given in the following paper. In the angle of isocline determinations, the light coming out of the glass bottom (GB) passes directly into the analyzing nicol (AN). This nicol for these experiments is attached in crossed position to the movable polarizing nicol (PN) through a connecting rod. Any rotation given to the analyzer (AN) is therefore simultaneously exerted on the polarizer (PN). Before the nicols are connected, they must be brought into the 90° position with respect to each other, by using the half-shadow (HS). This is a half-shadow wedge with variable angle of half-shadow after Macé de Lépinay manufactured by E. Leitz, Wetzlar. With this half-shadow the adjustment of the nicols into 90° position is very delicate. The position of the analyzer (AN) can be read by means of a graduated scale, to which a vernier was attached allowing direct readings to 0.05°. This scale, which is attached to the analyzer, is graduated on the lower surface and can be read conveniently with the aid of a concave mirror,[3] with illuminating device attached to it.

The objective (O) can be introduced by means of a dove-tailed slider on the same saddle stand on which the analyzing nicol (AN) is attached. The objective has a focal length, $f = 80$ mm., and is at a distance of almost twice the focal length from the bottom of the apparatus. The image of the two concentric cylinders, and of part of the annular gap filled with muscle globulin solution, is brought into the focal plane of the ocular (Oc) by means of the silvered reversing prism (P). The ocular is a microscope periplan of low magnification, with a cross-hair in the focal plane. The holder for ocular and prism is mounted on a special stand, not connected with the optical bench, and must be centered specially before starting a measurement. For the purpose of centering, the condenser C_3 is introduced. The lower surface of this condenser carries a concentric diaphragm which has an opening of $r = 0.075$ mm. The half-shadow wedge (HS) can be introduced

[3] A stock laryngological mirror with illuminating device was found convenient for the purpose.

immediately adjoining this diaphragm and marks with its fine line of separation a plane which contains the optical axis and is the plane of the drawing, Fig. 5.   The vertical cross-hair of the ocular is adjusted so that it coincides with the image of this line of separation of the half-shadow wedge.   The horizontal cross-hair is then set so that the small round image of the diaphragm is divided into four equal parts by the two cross-hairs.   The optic axis of the whole system is now indicated by the intersection of the cross-hairs, and the plane of reflection of the prism coincides with the plane of drawing in Fig. 5.

Once the optical system is centered, the concentric cylinders must be set in a definite position with respect to its axis.   First, the axis of rotation must be brought into the plane which is indicated by the horizontal cross-hair in the ocular.   This can be effected by observing a small mark on the bottom of the inner cylinder which had not been removed after turning.   The image of this mark represents the position of the axis of rotation, the inner cylinder being concentric with respect to the rotating outer cylinder, and is brought into coincidence with the horizontal cross-hair, by moving the apparatus as a whole.   Next, the concentric cylinders are so adjusted that the optic axis passes through the annular space at a given distance from the inner cylinder.   A sliding mechanism (not shown in Fig. 5) serves this purpose.   The cast-iron stand instead of resting directly on the wooden bench (B), is placed on two brass plates.   The upper plate slides on the lower by means of two grooves which are fitted to rails on the lower plate.   The direction of displacement is parallel to the plane indicated by the horizontal cross-hair and the amount of displacement is measured with a micrometer screw directly to 0.01 mm. Once the axis of rotation of the concentric cylinders is brought into coincidence with the horizontal cross-hair, further displacements by means of the two sliding brass plates cannot disturb this adjustment.   The image of the edge of the inner cylinder is then made to coincide with the intersection of the cross-hairs, indicating the optic axis, and the micrometer screw is read.   The micrometer screw is then advanced a given amount, producing displacement of the apparatus on the gliding mechanism and bringing the optic axis a given distance into the annular space.   With Cylinder 1, the displacement is 5.3 mm., the optic axis falls therefore on a

circle with the radius $r = 1.44$ cm. with respect to the axis of rotation. For Cylinder 2, the displacement is 4.0 mm., correspondingly $r = 1.70$ cm. These adjustments are necessary in order that different measurements may be made comparable.

Measurement of Angle of Isocline.—The angle of isocline is by definition the larger of the two angles, which the cross of isocline forms with the crossed planes of polarization of the polarizer and the analyzer. Two methods can be used to measure this angle. The first method, employed by Freundlich, Stapelfeld, and Zocher consists in measuring the angle at which the cross of isocline appears, keeping the position of the nicols fixed. The second method, which is used in this investigation, consists in rotating the nicols (keeping them crossed) until the cross of isocline is brought to a given position. The measurements are made only on one arm of the cross of isocline, since in the ocular (Oc) only an enlarged image of a small part of the annular space is visible. The visible arm of the cross of isocline is therefore brought to coincide with the horizontal cross-hair, by rotating PN and AN, simultaneously, with the connecting rod. The angle of this setting is read. It remains to determine the angle reading at which one of the planes of polarization coincides with the horizontal cross-hair. This angle can be determined by introducing a quartz wedge in place of the half-shadow wedge (HS) in such a way that its optic axis is parallel to the horizontal cross-hair. The angle of isocline Ψ is given by the difference between the two readings. The measurements of Ψ are as a rule averages of from ten to twenty settings. The experimental error is estimated at 1 per cent for concentrated solutions and average angular velocities. The setting is considerably less distinct and more diffuse in dilute solutions and at small angular velocities; for these solutions the error may be as much as 2 to 3 per cent.

The muscle globulin solution produces a slight but measurable rotation of the plane of polarization. This effect must of course be compensated as well as possible under the conditions. The setting of the nicols in crossed position is therefore always made while the solution is in the cylinder. The planes of polarization of the two nicols are therefore not at 90° to each other, but contain a correction due to the rotation in the muscle globulin solution.

All observations were made in green light, using the Eastman

Wratten filters Nos. 44 and 62 together. These together filter out almost all light which is not close to 540 to 550 $m\mu$. The experiments showed that this range is small enough for the accuracy of the measurements.

The Experiments.—In experiments on the angle of isocline in V_2O_5 sols, Freundlich, Stapelfeld, and Zocher (15) and Freundlich, Neukircher, and Zocher (13) found that one of the most outstanding properties of the angle of isocline was its change from 45° to 90° with the age of the preparation, described as "ageing effect."
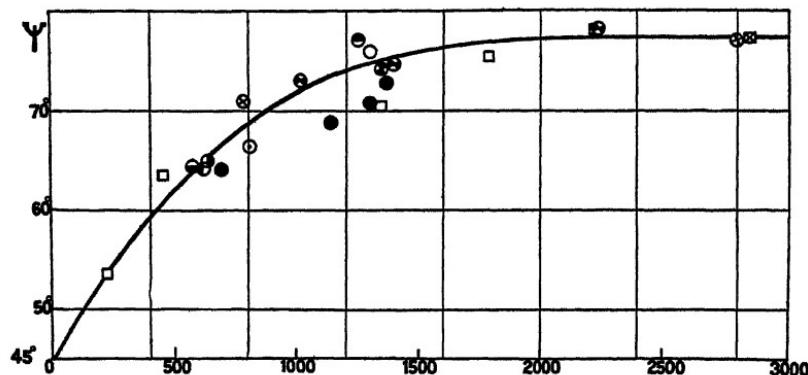
FIG. 6. Isocline measurements on preparations taken at the same velocity gradient and at constant temperature plotted against the concentration. The following preparations were investigated in Cylinder 1: C.M.G. (cow muscle globulin) VII o, C.M.G. IX ⊗, C.M.G. X₂ ◐, C.M.G. X₃ ◑, C.M.G. XI ◒, C.M.G. XIV ◓, C.M.G. XXI ●, C.M.G. XXII ⊙, C.M.G. XXIIB ⊕, R.M.G. (rabbit muscle globulin) III, ◔ ; in Cylinder 2, Preparation R.M.G. III □, R.M.G. IV ⊠.

The muscle globulin solutions were therefore studied very carefully with respect to age in order to discover such an effect. As far as our observations went (*i.e.*, as long as a single preparation could be kept free of bacteria and denaturation) no change in the angle of isocline could be observed. A preparation of muscle globulin gave the same angle of isocline immediately after preparation, as it did a month later. If a change occurred, it was invariably a loss of the property of producing double refraction of flow, accompanied therefore by disappearance of the cross of isocline altogether. In no instance could an increase of the effect during standing or

ageing be detected. All preparations gave an angle of isocline, according to their concentration, between 65° and 78°. This constancy was so striking that we were inclined to believe during the first experiments, that the angle of isocline was constant for all preparations. Later on it was found however that variations in concentration, angular velocity, and temperature produced slight but reproducible changes in the angle of isocline. The results of the isocline measurements on twelve different preparations, all taken at the same velocity gradient and at constant temperature 2–4° are plotted against the concentration, as determined by Kjeldahl nitrogen analysis, in Fig. 6.

Most of the measurements were made at the same angular velocity with Cylinder 1. Some of the points, as indicated, have been measured with Cylinder 2. Since the angle of isocline varies, especially in dilute solution, with the velocity gradient, these measurements had to be reduced to the same velocity gradient as that produced with Cylinder 1. The velocity gradient for a liquid of normal viscosity, between two concentric cylinders of radii R_1 and R_2 rotating with the angular velocity ω at a given point with the radius r, is given by:

$$\frac{dv}{ds} = r\frac{d\omega}{dr} = 2\,\Omega\,\frac{\dfrac{1}{r^2}}{\dfrac{1}{R_1^2} - \dfrac{1}{R_2^2}} \tag{1}$$

The angular velocity in all the measurements with Cylinder 1 was 95 R.P.M. or $\Omega = \frac{95}{60}\cdot 2\pi = 9.95$ sec^{-1}. All observations were made at the point $r = 1.44$ cm.; for $R_1 = 0.91$ cm., $R_2 = 2.0$ cm., the velocity gradient is 10.03. From this the angular velocity Ω' can be calculated, at which the same velocity gradient is obtained for Cylinder 2, at the given point $r' = 1.70$ cm. From $R_1' = 1.30$ cm. and $R_2' = 2.0$ cm., Ω' is obtained; $\Omega' = 4.97$ sec^{-1}. The validity of Equation 1 being assumed, the velocity gradient at the point of observation, Cylinder 2 being used, is twice as great as at the corresponding point with Cylinder 1. Or, the angular velocity with Cylinder 1 must be twice as great as with Cylinder 2 if the measurements with both cylinders are to be made under the same conditions with respect to the mechanical field of force; *i.e.*, the same

velocity gradient.   The points with Cylinder 2 in Fig. 6 represent measurements of the angle of isocline interpolated from the curves of Fig. 7 for an angular velocity $\Omega = 4.97$ sec^{-1} or 47 R.P.M. Since the applied corrections amount to 4 per cent at the maximum, the use of Equation 1 is justified, although it will be shown that muscle globulin does not fulfil the underlying assumption; $i.e.$, Newton's differential equation of flow.

The points in Fig. 6 do not fall very well on the curve which has been tentatively drawn.   The scattering of the points is far greater than the experimental error in the measurements of Ψ on any one preparation.   Hence this scattering must be due to variations
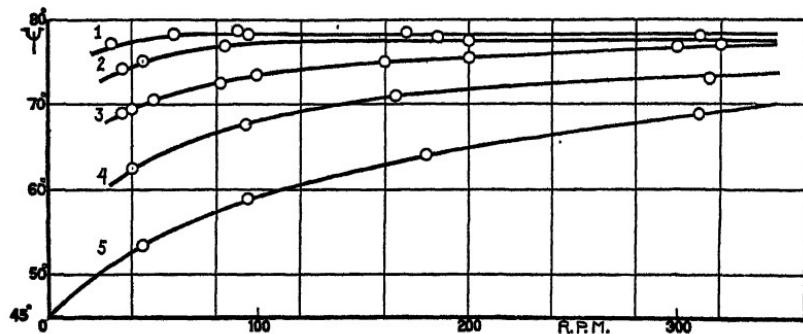
FIG. 7. The angle of isocline Ψ is plotted against the speed of rotation of the external cylinder for various concentrations of a preparation from a perfused rabbit.   Curve 1, concentration 2240 mg. of N per liter, η'_{rel}. 16.5; Curve 2, concentration 1790 mg. of N per liter, η'_{rel}. 10.5; Curve 3, concentration 1345 mg. of N per liter, η'_{rel}. 6.5; Curve 4, concentration 450 mg. of N per liter, η'_{rel}. 2.2; Curve 5, concentration 225 mg. of N per liter, η'_{rel}. 1.48.

between one preparation of globulin and another, not to variations in the measurement of Ψ.   The error in the Kjeldahl nitrogen analysis is estimated at 2 per cent and is insufficient to account for the deviations.   The Kjeldahl analysis, however, gives the total protein nitrogen; that of the optically anisotropic together with that of the inert proteins which might be present as impurities. Double refraction of flow is a property not found in many proteins and it is conceivable that this property is restricted to one definite protein within the muscle globulin preparation.

It might also be argued that the muscle globulin actually contains but one definite protein.   In this case the fluctuations of Ψ

would be due to the presence of denatured material formed during the preparation in quantities at present unknown. That this denatured material must have been formed during preparation and not afterwards follows from the observation that one given muscle globulin solution always gives the same angle of isocline during its "lifetime." This explanation gains further support

TABLE I.

Preparation XIV.

Concentration, 1247 mg. N per liter; temperature, 2°; Cylinder 1. Muscle globulin dissolved in potassium phosphate buffer, pH 6.8.

Ω	Ψ	Ω	Ψ	Ω	Ψ
	degrees		*degrees*		*degrees*
10.57 sec.$^{-1}$	78.2	20.47 sec.$^{-1}$	77.2	33.70 sec.$^{-1}$	76.6
	78.0		77.5		76.7
	77.4		76.2		78.0
	76.2		77.1		76.6
	77.3		76.1		76.6
	77.2		77.0		76.6
	77.4		78.1		78.1
	76.9		77.8		77.2
	76.4		77.5		76.8
	77.4		77.7		77.1
	78.3		77.9		76.6
	77.5		77.9		77.2
	77.7		77.8		77.2
	75.9		76.5		77.5
	77.6		77.0		
	77.7		78.2		
	77.5		76.3		
	78.2		76.4		
	78.5		76.6		
	76.1		76.2		
Average....	77.37		77.30		77.06

through the observation that all reagents which according to Anson and Mirsky (2) bring about denaturation cause disappearance of the double refraction of flow. Any foreign nitrogenous substance, or any denatured portion of the muscle globulin solution apparently does not participate in the production of the angle of isocline, whereas the figures of the Kjeldahl analysis

yield the total nitrogen, and by inference the total protein concentration. From this point of view it follows that if muscle globulin could be satisfactorily purified, the relation between the angle of isocline and the protein concentration would be reproducible.

The angle of isocline which is found more or less consistently for muscle globulin solution corresponds to the angle Ψ which is found in V_2O_5 sols of intermediate age. The behavior of the latter with respect to the Ψ-concentration relation is, however, different. For small concentrations (less than 0.015 per cent) the angle of isocline is constant, for higher concentrations it increases irregularly from 57° to about 73°, as Freundlich, Neukircher, and Zocher (13) found. It must be remembered however that the age of the V_2O_5 sol exerts a strong influence on Ψ and that is is almost impossible to eliminate this factor in a study of the Ψ-concentration relation in such a system. The behavior of muscle globulin in this respect is in marked contrast to that of the V_2O_5 sol.

Increasing the angular velocity in our experiments produced no effect on the angle of isocline in concentrated solutions, and for angular velocities higher than 9.5 sec^{-1}. The readings of the angle of isocline for cow muscle globulin Preparation XIV are reported in Table I.

It is apparent that within the experimental error, Ψ does not change with increasing velocity at this concentration.

For dilute solutions and low angular velocities a definite decrease of Ψ from the limiting value of 77° to 78° is observed. In Fig. 7 the angle of isocline, Ψ, is plotted against the speed of rotation of the external cylinder for various concentrations of Preparation R.M.G. III from a perfused rabbit.

The measurements include the lowest values of concentration and angular velocity that would still allow a definite reading of the angle, Ψ. Further dilution, or further slowing up of the rotation of the external cylinder, resulted in the disappearance of the cross of isocline to such an extent that no definite reading could be obtained. The extrapolation of the curve of the most dilute sample seems to point to an angle $\Psi = 45°$ for an angular velocity of 0, and it may well be possible that the other curves have their origin at the same point. The cross of isocline is, as a rule, a cross with straight arms, as drawn in Fig. 2. At low concentrations

however, the arms are twisted in such a way that close to the inner cylinder Ψ is largest, close to the outer cylinder smallest. The curvature in extreme cases corresponds to almost 10° difference in the isocline readings taken at these two points. The twisted isocline appears only at low concentrations and angular velocities, where considerable changes of the value of Ψ with changing angular velocity occur. At higher concentrations and at intermediate and high angular velocities the arms of the cross are straight.

Without treating here fully the mechanism of the cross of isocline, which is complicated and will be dealt with in the discussion of the results, the following simple explanation of this phenomenon as well as of the curves of Fig. 7 may be tentatively given.

The shearing forces, which develop between two laminæ of the muscle globulin solution during the rotation of the outer cylinder, are largely responsible for the orientation of the anisotropic muscle globulin particles. If muscle globulin were a fluid of normal viscosity, the shearing forces would be directly proportional to the viscosity of the fluid and (for a given apparatus) to the angular velocity of the outer cylinder. Increase of the angular velocity as well as increase in the concentration, i.e. viscosity, means therefore increase of the shearing force between two laminæ. This relation holds exactly for fluids with normal viscosity, and is probably in its general character also applicable to such solutions as muscle globulin which show anomalous viscosity (see below). Increasing values of the abscissa in Fig. 7 as well as of the parameter, concentration-viscosity, both indicate an increase in the shearing forces in the muscle globulin solution. It is therefore apparent that when the shearing forces have reached a certain strength, no further change in the angle of isocline occurs. At high and intermediate concentrations of muscle globulin solutions, the viscosity is such that with angular velocities higher than 90 r.p.m. the shearing forces are greater than those necessary to bring the angle of isocline to this limiting value. At these concentrations the cross of isocline has straight arms, which means that throughout the fluid the shearing forces are large enough to give the limiting value.

In order to understand why the arms of the cross of isocline appear twisted in some cases it is necessary again to fall back on

the case of a fluid with normal viscosity and to argue by similarity for the anomalous case.   The shearing force is, among other factors, proportional to the velocity gradient between concentric cylinders, is largest close to the standing (inner) cylinder, and smallest near the rotating (outer) cylinder.   (An excellent summary of these relations may be found in Hatschek's book (16)). The shearing forces are likewise larger near the inner cylinder and smaller near the outer cylinder.   It is deduced from Fig. 7 that smaller shearing forces produce smaller angles of isocline, provided the angles are smaller than the limiting value beyond which no
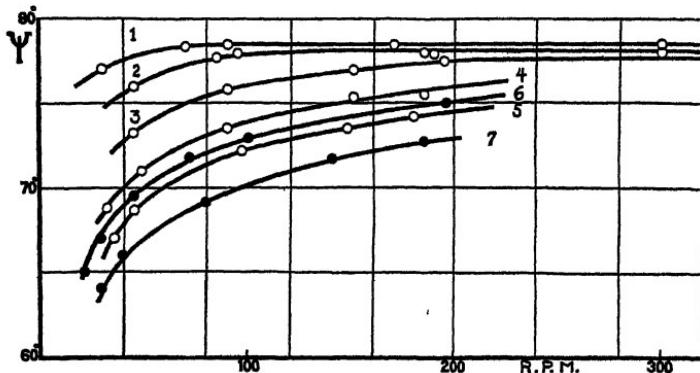
FIG. 8. Temperature effect on the angle of isocline of the same preparation represented in Fig. 7.   Curve 1, $t\,2°$, concentration 2240 mg. of N per liter; Curve 2, $t\,10°$, concentration 2240 mg. of N per liter; Curve 3, $t\,20°$, concentration 2240 mg. of N per liter; Curve 4, $t\,30°$, concentration 2240 mg. of N per liter; Curve 5, $t\,37°$, concentration 2240 mg. of N per liter; Curve 6, $t\,20°$, concentration 1340 mg. of N per liter; Curve 7, $t\,30°$, concentration 1340 mg. of N per liter.

further change occurs.   The angle of isocline near the outer cylinder should in such cases be smaller than near the inner cylinder, a deduction which agrees with the observations.   It must be remembered that the measurements of Fig. 7 were, of course, always made at the same point in the annular space, and are therefore not influenced by the effects of curvature.

The influence of temperature on the angle of isocline of the same preparation has been studied and the results are graphically represented in Fig. 8.

A similar set of characteristic curves is obtained and it appears

that the change in viscosity, which is the common factor in the experiments represented by Figs. 7 and 8, accounts to a large extent for the similarity in the curves.

The changes of the angle of isocline with varying pH, salt concentration, ionic strength, and other factors have not yet been extensively studied. Such a study necessitates further purification of the globulin so that all preparations become reproducible with respect to the concentration of the protein producing double refraction of flow. A satisfactory method of purification has not yet been devised and the study of the angle of isocline with respect to these factors has therefore been postponed.

That muscle globulin is not normal with respect to viscosity has already been mentioned. Like most doubly refractive colloids the solution exhibits a property which has been described as "rigidity" (Fliesselastizität). Qualitatively this property can be detected by determining the rate of flow of the solution at different pressures in a capillary viscosimeter. Normal fluids give in these measurements a coefficient of viscosity which is independent of pressure; rigid fluids show with increasing pressure a characteristic decrease of the viscosity towards a limiting value. For the quantitative study of this effect the capillary viscosimeter is unsuitable, since the pressure on the fluid varies continuously during any one experiment. An apparatus of the Couette-Hatschek[4] type should be employed (see Hatschek (16)). But even with this instrument it has not yet been possible to obtain a definite modulus of rigidity for such fluids and the description must remain, for the present, qualitative.

Certain preliminary viscosity measurements were undertaken in order to determine in how far one was justified in classifying muscle globulin as a rigid fluid. Some of the viscosity data are tabulated in Table II. The apparent viscosities η' are relative viscosities, the outflow time of muscle globulin being compared

[4] An attempt was made to use the inner cylinder in this investigation as a Couette-Hatschek viscosimeter and a correspondingly built cylinder was tried out. Owing to the rather small dimensions of the complicated mechanism, and to difficulty with the platinum plating and the proteins, no reproducible results have so far been obtained. A thorough investigation of the viscous anomalies in proteins in general with the aid of a properly constructed apparatus remains highly desirable.

with the outflow time of distilled water in the same Ostwald viscosimeter and at the same pressure. The pressure-head is kept constant by a large reserve volume (5 liters) and a valve, consisting of a tube submerged in a water column of varying height. The pressure is conveniently regulated by this valve, and read by means of a water manometer. In a study of the rigidity of gelatin solutions Freundlich and Abramson (12) found a very marked alteration of this property with the age of the solution. Such an ageing effect has not so far been detected in muscle globulin. The time of outflow of a muscle globulin solution, which shows a constant angle of isocline, is found to be independent of the time which the solution remains in the viscosimeter

TABLE II.

Relative Viscosities of Muscle Globulins.

C.M.G. XII.		R.M.G. III, concentration 1.0.		R.M.G. III, concentration 0.6.		R.M.G. III, concentration 0.1.	
P	η'	P	η'	P	η'	P	η'
cm. H_2O		cm. H_2O		cm. H_2O		cm. H_2O	
10.0	10.8	10.0	16.5	9.6	6.51	9.6	1.48
24.7	10.15	16.5	16.5	13.3	6.49	11.6	1.48
38.8	9.8	25.5	16.4	19.3	6.30	14.2	1.48
47.9	9.5	33.7	16.1	29.9	6.13	15.3	1.52
75.0	8.3	45.8	15.9	35.5	6.09	18.9	1.53
		65.7	15.1	45.2	5.93	27.1	1.49
		91.1	14.1	55.7	5.81	34.3	1.47
				70.4	5.51	38.7	1.45

previous to flowing for the first time. Repeated runs in the viscosimeter as well as standing in the cold room at $-2°$ had no effect. In this respect the absence of ageing agrees well with the absence of this effect on the angle of isocline and double refraction. For the reasons stated above these few experiments on rigidity cannot be considered final, and further investigations of this interesting property should be undertaken.

The rigidity of muscle globulin solutions has independently been confirmed by observations of one of the inner cylinders suspended, for the purpose, on a torsion wire and dipped into the solution. If the outer cylinder was rotated very slowly at about 0.1 R.P.M. (conveniently effected by attaching it to a slow kymo-

graph) the inner cylinder accurately followed the motion of the outer cylinder up to a given limit. At this limit a sudden jerk occurred. The motion is readily followed with the aid of a mirror, scale, and telescope. The change in velocity, manifested by a jerk, represents the moment when the elastic forces are overcome by the counteracting torque of the torsion wire, and a break occurs. The final equilibrium of the torsion cylinder is reached in several jerks, which is in evident contrast to the viscous behavior of any normal liquid.

Thixotropic Gel.—After repeated washing of the muscle globulin solution, the preparation becomes practically salt-free and forms
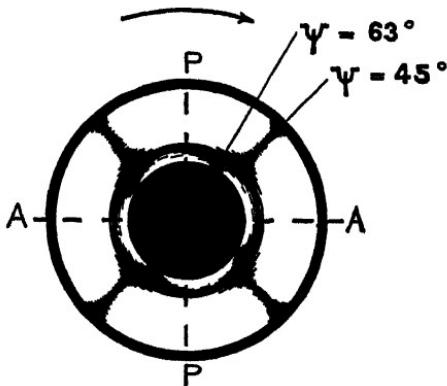
FIG. 9. The cross of isocline as it appears when the outer cylinder is set into permanent rotation in the thixotropic gel.

a clear gel even at an extremely low protein concentration (about 0.3 per cent). This gel has been described in the preceding paper by Edsall (8). Rabbit muscle globulin seems to pass into the gel state more readily than the muscle globulin of cows. Several of these gels were studied with respect to their angle of isocline in our apparatus. A small displacement of the outer cylinder, not exceeding the limit of elasticity of the gel, produces at once a very distinct cross of isocline at an angle $\Psi = 45°$. Under these conditions the gel may be regarded as a deformed elastic solid. Stokes' theory can therefore be applied and this angle is to be expected. The deformation produces photoelastic effect, which disappears as soon as the stress of the outer cylinder, acting against

the elastic forces of the gel, is released.   If the outer cylinder is set into permanent rotation entirely different conditions seem to prevail.   The cross of isocline, as it appears under these conditions, is sketched in Fig. 9.

The most striking phenomenon is a dark ring separating the two different crosses of isocline.   The outer cross has an angle of isocline of 45°, the same as the slightly deformed gel.   The inner cross however has an angle of about 61° to 63° not very clearly measurable, but definitely not 45°.   The black ring separating the two crosses of isocline proves to be isotropic.

The gel has other interesting properties.   By mere vigorous shaking it is transformed to the fluid state and sets again to a gel after a few minutes.   Gels which exert this property have been . called thixotropic (33) and a number of them are known.   The thixotropy of this gel gives a clue to its behavior in the concentric cylinder apparatus.   The shearing forces which arise in the apparatus in rotation have a tendency to destroy the gel structure permanently.   This destruction is most effective near the inner cylinder where the shearing forces are largest.   The neighboring layer around the inner cylinder is therefore in the fluid state and the isotropic ring indicates where the gel "breaks off."   From there on the gel moves as a solid block and gives an angle of isocline of 45°, due to the small elastic stress which is apparently still exerted on it, as a result of the frictional forces at the interface gel-fluid.   The fluid layer, however, gives an angle of isocline such as is found in muscle globulin *solutions* (of a similar small protein concentration) which is partly due to orientation of the anisotropic protein particles.   At the interface neither definite elastic deformation nor orientation occurs and isotropy must prevail.

This explanation was confirmed by casual observations that small particles in the outer solid block moved at the same angular velocity as the glass cylinder.[5]

A certain significance is attached to these observations with

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[5] Quartz particles suspended in the gel showed no Brownian movement and did not change their position.   The cataphoretic velocity of these particles, however, was that of quartz particles in a fluid.   These observations which Dr. H. A. Abramson very kindly made, confirm the thixotropic character of this gel.

respect to the behavior of the living muscle cell. Kühne (18) reported that he observed a nematode wandering through a muscle cell, and stated that the nematode had no difficulty in passing through a cross-striated muscle fiber. Where the animal touched the striations with its head, Kühne stated that they disappeared at once, and reappeared after the animal had passed. Freundlich (11) has suggested that the interior of the muscle cell is a thixotropic gel, which would explain this observation, quoted as a curiosity in many text-books. The thixotropic muscle globulin gel is of course far from representing the natural state of the protein in the muscle, but the fact that such a gel may be prepared from extracted muscle protein has a certain interest.

Discussion of Results.

The most striking property of the muscle globulin solution with respect to the measurements of the angle of isocline, is the reproducibility of the measurements with respect to age. In the V_2O_5 sols the angle Ψ changes from 45° for a very young sol to almost 90° for the very old sols. The angle of isocline in a muscle globulin solution corresponds to that found in a V_2O_5 sol of intermediate age, with the important difference that the behavior of the muscle globulin is independent of age. Freundlich, Stapelfeld, and Zocher (15) found that this ageing effect in the V_2O_5 sol was largely due to growth of the needle-shaped V_2O_5 particles, which grew longer and longer as the preparation became older. This observation has been confirmed by many other independent observations. (Errera (9) for instance found increasingly abnormal dielectric constants with increasing age of a V_2O_5 sol, which he ascribed to the increasing length of the V_2O_5 needles.) It appears therefore that the angle of isocline is closely related to the shape of the anisotropic particles and that increasing asymmetry results in an increasing angle of isocline from $\Psi = 45°$ towards $\Psi = 90°$. Since in muscle globulin no such change with age has been observed the conclusion may be drawn that the shape of the anisotropic muscle globulin particles is the same, at least for any one preparation, and probably for all preparations thus far studied.

This apparent constancy of the shape of the particles might be explained in either of two ways. Either the anisotropic protein

in muscle globulin solutions is made up of particles of uniform and constant shape (monodispersity) or the particles have different shapes and the "average shape" remains constant in a preparation (polydispersity).   If the second assumption were correct, it would be hard to conceive why it is, that for twelve different preparations (Fig. 6) practically the same angle of isocline was found, which would mean that the average shape in each preparation was the same.   Furthermore it seems improbable that the formation of larger and smaller particles, which at some stage has been possible, should come suddenly to a standstill, as it must if the average shape is to remain constant.   It therefore appears reasonable to accept the assumption that the protein responsible for double refraction in muscle globulin is monodisperse.   This would explain both the constancy of the angle of isocline with respect to age, and the reproducibility of different preparations.

The assumption that the anisotropic protein is monodisperse is in agreement with the general evidence that by suitable preparation certain proteins can be obtained in monodisperse form.   The general evidence that certain proteins can be prepared as chemical individuals has been accumulating in recent years and has been confirmed in the last few years by Svedberg and his collaborators on the basis of their measurements with the ultracentrifuge.   In a recent publication Svedberg (32) has given a list of the molecular weights of proteins which so far have been obtained in monodisperse form.   Muscle globulin is not mentioned in this list but is listed among fourteen other proteins as being polydisperse.   Svedberg however ascribes this rather to a deficiency in the method of preparation than to a property inherent to these proteins.   In this connection it may be pointed out that the method of preparation of muscle globulin in our experiments has been carried out with certain added precautions which proved necessary in order to obtain reproducible results (8).   Very probably the optical method employed in this investigation only detects effects produced by the anisotropic particles and is uninfluenced by the presence of small amounts of foreign or denatured protein.   This together with the new precautions in the method of preparation may well account for the discrepancy between the results with the ultracentrifuge and with the double refraction apparatus.

The fact that variations in concentration, angular velocity,

and temperature produce slight but reproducible changes in the angle of isocline makes it apparent that the shape of the particle is not the only factor determining the angle of isocline. Superficially the relation between the shape of the particle and the angle of isocline appears to be simple, but even in the present state of knowledge an accurate evaluation of all factors involved in isocline formation would be very complicated. No attempt toward a solution of this problem from the theoretical side has yet been made. What follows, therefore, is a general descriptive exposition of our views, which have been largely influenced by those previously presented by Freundlich, Neukircher, and Zocher (13).

The complicated behavior of muscle globulin can be approximately described in terms of two ideal cases.

As a first simplification it may be assumed that the annular space between the two concentric cylinders is filled with a pure liquid of considerable viscosity. From the experiments of Vorländer and Walter (35) it is known that in a small annular space and at very high angular velocities (up to 2000 R.P.M.) such a liquid may show double refraction. The amount produced is largely determined by the chemical structure of the molecule and, in the case of homologous series, is definitely related to the length of the carbon chain. The angle of isocline has always been reported to be 45°. From this Raman and Krishnan (26) have concluded that the property of molecular double refraction of flow is shown only by fluids which consist of anisotropic, asymmetric molecules. Orientation of these molecules is produced by the shearing forces which act, according to the theory of Stokes, (Fig. 3) in the form of stresses and pressures at 45°. If it can be assumed that the axes of optical and geometrical asymmetry coincide, an angle of isocline of 45° would result. On this basis, agreement between theory and experiment exists. Molecular double refraction of flow is according to this theory an orientation effect of anisotropic molecules. It can be produced only by an intense mechanical field, which necessitates high viscosity of the fluid, high angular velocity of the rotating cylinder, and small annular space. The old "elastic" theories, which were promoted by Natanson (24) and others, never succeeded in accounting for the experimental data.

As a second simplification it may be assumed that the annular

space is filled with a gel of a substance which in the fluid state shows no double refraction in a moderate mechanical field.   Deformation of such a gel by a small torsion of the outer cylinder results in the production of photoelastic effect.   According to Stokes the cross of isocline appears, under these circumstances also, at 45°.   It must be noted, however, that the shearing forces which are the same in both cases act differently.   In the viscous fluid they produce orientation of anisotropic molecules, in the gel they produce photoelastic effect on the basis of a mechanism which is not yet clearly understood.   The fact that both of these extreme cases result in an angle of isocline of 45° is partly responsible for the still wide-spread belief that the double refraction in viscous fluids is a photoelastic effect in the sense described for the ideal gel.   As long as the exact nature of the photoelastic effect remains unknown, these two extreme cases must be considered separately. The molecular double refraction of flow should be considered as an orientation effect, and the accidental double refraction in a gel as photoelastic effect.

The double refraction of flow, which is generally found in colloids, contains both of these extreme ideal cases.   This renders their behavior complicated.   Double refraction of flow in colloids, as in molecular solutions, is largely due to orientation of anisotropic elements.   It might be questioned whether the orientation of an anisotropic micelle can be compared with the orientation of an anisotropic molecule.   It is quite probable, however, that the difference is primarily a question of size.   In accordance with this view it is evident that in colloids even a very small mechanical field is sufficient to bring about orientation, whereas for pure liquids very high forces are necessary.   The very young V_2O_5 sols illustrate this resemblance.   They are almost normal with respect to viscosity and their micelles are small; they are found, like pure liquids, to give an angle of isocline of 45°.   With ageing they exhibit a new property described as "rigidity" (Fliesselastizität).   The viscosity of these sols is no longer normal since they develop elastic forces under the influence of deformation. In this respect, though in liquid form, they manifest the properties of solids.   On the basis of considerations advanced many years ago by Schwedoff (30), Freundlich, Neukircher, and Zocher (13) have shown that such elastic forces may produce deformations of

the oriented anisotropic particles so marked that angles of iso-cline between 45° and 90° result, according to the strength of the elastic deformation.

Such elastic forces can be studied qualitatively by measuring viscosity as a function of pressure in the capillary viscosimeter, or as a function of angular velocity in an apparatus of the Couette type. Fluids with normal viscosity give viscosities independent of the magnitude of the shearing stresses. Fluids which show rigidity give "apparent viscosities, η'" which increase to enormous values if the shearing stresses employed for the measurement are maintained sufficiently small. Although the Couette viscosim-ter (especially in the form given by Hatschek (16)) is far better suited to the study of these elastic forces than any other instru-ment thus far described, it has not yet been possible to character-ize these elastic forces quantitatively in a satisfactory way. (An excellent review of these phenomena may be found in Hatschek's book on viscosity (16), Ostwald has given a comprehensive survey of all the formulæ which have been developed (25), and a mathe-matical theory has recently been advanced by Reiner and Riwlin (28) and Reiner (27).)

The measurements and observations on the apparent viscosity of muscle globulin show that these solutions also show effects of rigidity. If such effects did not exist an angle of isocline $\Psi = 45°$ would be expected for the oriented muscle globulin particles.

In this connection a possible explanation of the angle of 78° must be mentioned, which is related to certain physiological ob-servations which have often been quoted.

It might be possible that the orientation of the anisotropic muscle globulin particles actually takes place at 45°, but that their optic axis is at 33° to the geometric axis. As a result the angle of isocline would appear at 78°. Such deviation is possible, and must be considered in the absence of definite contrary proof. The optic axis of the muscle fiber coincides, however, with the long axis of the fiber. Since it will be shown that the anisotropy of the muscle globulin particle can account, to a large extent, for the anisotropy of the whole muscle fiber, it would be necessary for the muscle globulin particles to be arranged in the muscle fiber with their long axis on a helicoid of 57° to bring their optic axes into coincidence with the long axis of the fiber. Meigs (21)

has reported a beaded structure of the cross-striated muscle fiber which might conceivably represent a helicoid. That muscle globulin shows an angle of isocline at 78° might be considered evidence for the helicoid hypothesis. In this connection renewed importance might be attributed to Bernstein's (1916) observation that the artificial contractions of catgut upon swelling, which Engelmann (1893) described, were due to the helicoid texture of these fibers.

Measurements of the apparent viscosity of muscle globulin solutions have shown, however, that they exert rigidity as do V_2O_5 sols of intermediate age, and it seems therefore simplest to assume that the deviation of the angle from 45° is due to the same elastic deformation, which has been made responsible for the angle of isocline deviations in V_2O_5 sols. The helicoid hypothesis appears improbable in the light of evidence to be presented in the following paper.

The experimental facts are best described at present by ascribing the cross of isocline primarily to the orientation of asymmetric, anisotropic muscle globulin particles of constant shape and size, brought about by shearing forces acting at 45°. In addition elastic forces between these elements add to the viscous shearing forces and produce a deformation of the particles, resulting in an angle of isocline of 78°. These hypotheses are unquestionably over simplified. Concentration, angular velocity, and temperature are factors which alter the magnitude of these elastic forces in a complicated manner. In very dilute solutions where viscosity measurements yielded no evidence of such elastic forces, the angle of isocline was found to approach 45° for very small angular velocities. How closely the viscous and elastic forces in such a solution are related to the shape and size of the particles, and how far they are influenced separately by temperature, concentration, and angular velocity must await further investigation.

SUMMARY.

1. Muscle globulin solutions show double refraction of flow. This effect is ascribed primarily to orientation of anisotropic particles and secondarily to photoelastic phenomena. The black cross which appears in polarized light in the solution in a rotating cylindrical apparatus is called the cross of isocline (Wirbelkreuz)

and the angle at which the arms of the cross appear, the angle of isocline (Kreuzwinkel).

2. The apparatus for the determination of the angle of isocline and the double refraction is described.

3. The angle of isocline of muscle globulin solutions is independent of age. For intermediate and high concentrations at low temperature (2–4°) it is always between 77.5° and 78.5°. For small concentrations it decreases with dilution and approaches 45° as a lower limit.

4. For constant temperature and varying concentration characteristic curves are obtained for the relation between the angle of isocline Ψ and the angular velocity Ω.

5. For constant concentration and varying temperature similar curves are obtained for the Ψ-Ω relation.

6. The muscle globulin solution is not normal with respect to viscosity, but possesses rigidity. However no effect of age has been found.

7. Muscle globulin gels have been studied. They are found to be thixotropic and accordingly yielded a more complicated cross of isocline.

8. The measurements of angle of isocline have been interpreted as an indication that the muscle globulin particles are of uniform shape and size (monodispersity).

9. The complicated mechanism of the formation of the cross of isocline is discussed, and attributed to the orientation and deformation effects produced by the viscous and the elastic forces in the solution, acting on the anisotropic muscle globulin particles.

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STUDIES IN THE PHYSICAL CHEMISTRY OF MUSCLE GLOBULIN.

IV. THE ANISOTROPY OF MYOSIN AND DOUBLE REFRACTION OF FLOW.

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A beam of plane-polarized light, on passing through contractile tissue, is changed into elliptically polarized light. The contractile tissue is doubly refractive. According to Engelmann this is a fundamental property, all contractile tissues showing double refraction. Double refraction does appear also in non-contractile tissues, but it is found invariably if the tissue is contractile.[1] The study of this optical property, and of the factors which underlie it, may therefore be expected to throw some light on any theory of muscular contraction. Even if the double refraction is not directly involved with the cardinal factors producing contraction, the fact that it undergoes sudden definite changes at the onset of contraction would suggest that it serves as a valuable indication of certain underlying phases of the contractile process.

In cross-striated muscle, double refraction is restricted to the so called anisotropic disc Q, and to a smaller extent to the narrow band Z. The anisotropic disc Q behaves optically like a uniaxial positively doubly refractive crystal, whose axis lies parallel to the axis of the fiber. This property of the anisotropic disc has long been known. Brücke (1858) attempted to explain the facts by assuming the presence of small uniaxial positive doubly refrac-

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[1] Double refraction and contractility appear simultaneously in the muscles of the embryo; and when muscle is transformed into functionally different tissue (as in the formation of the electric organ in the ray) the first sign of the change is the disappearance of double refraction. See the excellent book of Schmidt (26).

tive crystals, imbedded in the anisotropic disc, and oriented with their axes parallel to the axis of the fiber. He named these hypothetical crystals "Disdiaklasten" and regarded them as being of submicroscopic size.

In the following generation, unfortunately, the theory of Disdiaklasten was generally discarded by physiologists in favor of von Ebner's theory of internal stresses. Von Ebner attributed double refraction in tissues entirely to accidental double refraction, or photoelastic effect (a brief discussion of this effect is given in the preceding paper (21)). The internal stresses necessary to produce such a condition in the muscle fibers were ascribed to growth and expansion of the tissue cells. But very large internal stresses were required to account quantitatively for the observed double refraction, and no evidence was produced that stresses of this magnitude could arise in the cell. Nevertheless, von Ebner's theory was almost generally accepted for 20 years. Finally the work of Ambronn and his school (1) proved definitely the existence of crystalline micelles (in the sense of Nägeli) as the underlying factor producing double refraction in most tissues. The theory of Disdiaklasten was revived.

Bottazzi and Quagliariello (4) suggested that the ultramicroscopic granules which they found in muscle press juice might represent the Disdiaklasten of Brücke. These granules, however, showed no sign of double refraction under the polarizing microscope, an observation since confirmed by Stuebel (27). The suggestion must therefore be considered hypothetical. Any tissue extract supposed to contain the Disdiaklasten should show double refraction under appropriate conditions. Without such proof, this interpretation remains inconclusive.[2]

The patterns obtained in x-ray spectrograms of muscle (Herzog and Jancke (14) and Clark (6)) have been held to support a theory like that of Disdiaklasten. These views must be considered with caution. The period of identity producing the pattern has been calculated by Herzog and Jancke to be 10 Å. on stretched fibers and by Clark to be 8.5 to 9.5 Å. for various fibers. The latter concludes that, this being too short a distance for the length of the long organic molecules involved, it may represent the width of

[2] The chemical relationship between the ultramicroscopic granules and the myosin which we have studied is discussed in a preceding paper (8).

the molecules lying side by side in equidistant planes. For the ordinary period of identity of proteins, fatty acids, and lipoids, much larger distances would be expected, and the significance of the x-ray patterns, as Meyerhof (20) has pointed out, remains at present uncertain. Possibly the further development of the method recently employed by Boehm and Schotzky (3) will throw new light on the problem. At present we believe that the x-ray patterns cannot be taken as convincing evidence of the presence of Disdiaklasten.

The existence of Disdiaklasten—the term being used in a somewhat broader sense than Brücke originally intended—has been beautifully shown by Stuebel (27). Stuebel's experimental work is based on the theory of "compound double refraction" developed by Wiener (33), and applied to a system termed by Wiener a "Stäbchenmischkörper." Such a system consists of a bundle of isotropic rods (refractive index n_1), small compared with the wave-length of light, all parallel, and immersed in a medium of refractive index n_2. Such a system taken as a whole is optically anisotropic, as Wiener showed, even if the components taken separately are isotropic. The resulting double refraction is always positive, and changes with the difference between the two refractive indices. The optic axis lies parallel to the bundles of rods. The amount of double refraction is given from the theory by the equation:

$$n_e^2 - n_o^2 = \frac{V_1 \, V_2 \, (n_1^2 - n_2^2)^2}{(V_1 + 1) \, n_2^2 + V_2 \, n_1^2} \tag{1}$$

V_1 and V_2 are the volumes of the respective components, n_1, the refractive index of the rods, n_2 that of the medium, n_e and n_o, the refractive indices for the extraordinary and the ordinary beam, respectively.

If the double refraction $(n_e - n_o)$ given by Equation 1 is plotted as ordinate against the refractive index, n_2, of the medium as abscissa (on the assumption that the medium can be altered without altering or injuring the structure of the rods) a parabola results, which is convex to the axis of the abscissa. The vertex of the parabola touches the abscissa at the point $n_1 = n_2$; for at this point $n_1^2 - n_2^2$ is zero, hence $n_e - n_o$ vanishes also.

If the rods of the system are in themselves optically anisotropic

(Eigendoppelbrechung) conditions are slightly altered. The whole parabola is displaced along the ordinate axis by an amount equal to the double refraction of the rods themselves. These deductions from Wiener's theory have been widely applied by Ambronn and his school, and have led to a deeper knowledge of the ultramicroscopic structure of many tissues.

In Fig. 1, the data obtained by Stuebel on cross-striated muscle have been plotted as above indicated. It is apparent that the experimental points lie on a parabola, in accordance with Wiener's theory, and the conclusions which Stuebel has drawn on this basis must be regarded as very significant. Briefly they are as follows: The anisotropic disc contains a great number of rod-
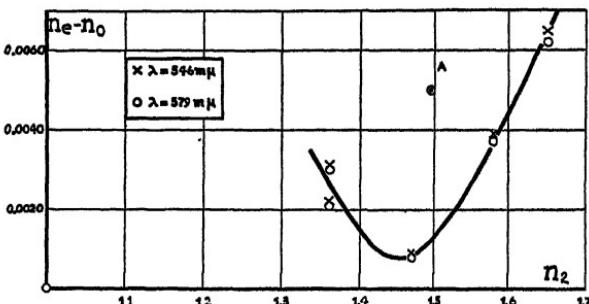
FIG. 1. Double refraction ($n_e - n_o$) of cross-striated muscle, as a function of the refractive index (n_2) of the medium. Plotted from Stuebel's data. The point A indicates rise of double refraction after lipoids are extracted from the muscle.

shaped particles (small compared to the wave-length of light) oriented with their long axes parallel to the axis of the fiber. The variation of $n_e - n_o$ with the refractive index of the surrounding medium is very strong evidence for this conception, and this Stäbchendoppelbrechung accounts for a large part of the double refraction in the intact muscle fiber. Moreover, the rods themselves are positively doubly refractive, since the parabola never touches the axis of the abscissa. If Stuebel treated the muscle with a lipoidal solvent before immersion, he obtained points similar to point A in Fig. 1, indicating that there is some negative double refraction due to the muscle lipoids. This component produces a depression of the positive double refraction arising from

the anisotropic rods and from their structural arrangement in the fiber. When the lipoids are extracted by a suitable solvent, the positive components are left free to exert their full effect, and the double refraction rises.[3]

It should be emphasized that Stuebel's picture of the fine structure of the anisotropic disc has a better claim to represent the facts than any apparent structures revealed by the microscope. These latter structures are all in size so close to the resolving power of this instrument that it is nearly impossible to distinguish them from artificial diffraction patterns, which become very disturbing at high magnification.[4] In view of this fact, apparent structures, such as those described by Lutembacher (19) cannot (without further evidence) be accepted as significant.

The muscle globulin solutions which we have prepared show marked double refraction of flow, as stated in the preceding paper. We interpret this as an indication that they contain the anisotropic rod-shaped particles from the doubly refractive disc of the cross-striated muscle. In the preceding paper some of the complicated mechanisms involved in double refraction of flow have been discussed. In this paper is presented the experimental evidence that muscle globulin solutions contain anisotropic particles, and that the anisotropy is connected with the chemical structure of the protein.

Apparatus.

The main features of the apparatus used in this study have been described in the preceding paper. To determine the anisotropy of the flowing particle, the amount of double refraction produced by a given solution must be accurately measured. For such

[3] Von Fürth's (10) diagrams of the hypothetical ultramicroscopic structure of the muscle fiber, though drawn several years before Stuebel's work and for an entirely different purpose, illustrate Stuebel's conceptions admirably. This does not imply that Stuebel's experimental data therefore support von Fürth's "acid-swelling" theory of muscular contraction. As Meyerhof ((20) p. 291) states in discussing Stuebel's work: "Es lässt sich daher jede Theorie der Kontraktion met diesen Befunden vereinigen. Nur das eine ergibt sich daraus, dass die Muskelfaser eine micellare Ultrastruktur besitzt, und die Wahrscheinlichkeit, dass der Kontraktionsvorgang sich an dieser Ultrastruktur vollzieht."

[4] See the first chapter of Bayliss's "General Physiology" for a brief nontechnical discussion of this point.

quantitative measurements, compensators are used. Plane-polarized light, on passing through a doubly refractive fluid (the plane of polarization being at 45° to the optical axis of the flowing fluid) is changed into elliptically polarized light. On entering the fluid, the original vibration is resolved into two plane-polarized components, vibrating at right angles to one another. These components travel through the fluid with different velocities, emerge with a certain phase difference, and compound into elliptically polarized light. The character of this elliptical polarization is determined by the phase difference Δp, the latter being given by the equation:

$$\Delta p = \frac{S}{\lambda_0} (n_e - n_o) \tag{2}$$

in which Δp is the phase difference, expressed in wave-lengths, n_e and n_o the refractive indices of the two components, S the distance traveled in the doubly refracting medium, expressed in the same units as the wave-length λ_0 of the incident light. (Those not familiar with these relations may be referred to a very brief and clear exposition recently given by Kunitz (16), or to Ambronn and Frey's book (1).)

This phase difference which has arisen between the two components may now be compensated so that plane-polarized light results again. If the compensator has previously been calibrated, the phase difference can be determined. Thence the amount of double refraction $n_e - n_o$ can be calculated, according to Equation 2, if the wave-length λ_0 of the incident light and the thickness of the layer S are known.

Sénarmont Compensator.—For the study of the double refraction produced by flowing muscle globulin solutions, a Sénarmont compensator has been used. (A simple description of this compensator is given in Ambronn and Frey's book (1); a recent treatment of the exact theory of its use has been given by Szivessy (30).) It consists of a mica plate $\left(\frac{\lambda}{4}\text{ in Fig. 5 of the preceding paper}\right)$ which produces a phase difference of a quarter wave-length for light within the range 540 to 550 $m\mu$, within the limits of accuracy set by the other sources of error in these investigations. (The light is kept within this range by the use of Eastman Wratten

filters Nos. 44 and 62 in combination.)   The mica plate is slid into the condenser carrier and can be rotated on a small revolving disc, until it is adjusted to the plane of polarization of the incident light beam.   This adjustment consists in setting it so that the direction of its largest refractive index coincides with the plane of polarization of the light from the polarizer.   If the muscle globulin solution is at rest, and the two nicols are crossed, insertion of the mica plate *in this position* produces no change in the field of vision, the light from the polarizer passing through the plate unaltered.   As soon as the muscle globulin is set into motion, the light emerging from the solution becomes elliptically polarized; and when it falls upon the quarter wave-length plate, definite changes are observed.   The elliptically polarized light is always transformed back into plane-polarized light in passing through the oriented mica plate which therefore *in this specific orientation* acts as a compensator provided that the long axis of the ellipse coincides with one of the planes of polarization.   This condition is always fulfilled if the planes of polarization are carefully set at 45° to the optic axis of the flowing solution.[5]   The resulting plane-polarized light, however, has been rotated by an angle $\Delta°$ relative to the plane of polarization of the light from the polarizer.   The angle Δ bears a simple linear relation to the phase difference Δp between the components of the elliptically polarized light:

$$\Delta° = 180 \cdot \Delta p = \frac{180 \cdot S}{\lambda_0} (n_e - n_o) \qquad (3)$$

If polarizer and analyzer were previously crossed, the analyzer must now be rotated through the angle $\Delta°$ to extinguish the plane-polarized light emerging from the quarter wave-length plate. The angle $\Delta°$ (and thereby the amount of double refraction produced by the flowing solution) is thus readily determined.   This

[5] Muscle globulin solutions show no dichroism; *i.e.*, the two components traveling through the solution with different velocities, are equally absorbed; their relative amplitudes therefore remain unaltered.   If muscle globulin solutions were dichroic, special care would be necessary to reorient the mica plate with respect to the long axis of the ellipse.   The angle of this reorientation would then give a measure for the amount of dichroism present.   However no trace of dichroism was detected in any of the muscle globulin preparations thus far studied.

method is simple and accurate for the determination of phase differences less than $\frac{\lambda_0}{2}$. Higher phase differences can only be measured by very careful observation of the compensating angles and interference colors, and for such measurements a Babinet compensator is preferable.

The accuracy of the compensator is considerably increased by the use of a half-shadow (*HS* in Fig. 5, preceding paper). The accuracy of a half-shadow method is determined by two factors, one purely physical, the other physiological. Physically the accuracy of the setting increases as the half-shadow angle between the two sides is decreased. Such a decrease, however, reduces the illumination of the field of vision. There is an optimal illumination zone within which the sensitivity of the observer's eye is at a maximum, and a decrease of illumination below this point reduces the ability of the observer's eye to detect small changes. For an incident beam of light of given intensity an optimum angle of half-shadow can be found, which is determined by both the factors mentioned above. This optimal angle varies with the intensity of the incident light, which in turn varies greatly with the concentration of protein in the solution under study. A half-shadow wedge with variable angle of half-shadow, of the type originally designed by Macé de Lépinay, and manufactured by E. Leitz, Wetzlar, was found best adapted for our purposes. By sliding the wedge back and forth in its container, and observing at different thicknesses of the wedge, an optimal adjustment could be made for any given solution.

By the aid of this half-shadow, the analyzer could be set (within the desired limits of accuracy) at 90° to the plane of polarization of the light emerging from the compensator. The experimental error of the setting was about ±0.4°. The percentage error therefore varied with the value of $\Delta°$, being smallest for large values of $\Delta°$. For small values the error was diminished by making up to twenty readings for a given setting, whereas for large values of $\Delta°$ only five readings were necessary. The setting of the half-shadow also was more sensitive when the solutions were dilute and the light emerging from the solution therefore more intense.

Before measurements of double refraction can be made, the optic axis (*i.e.* the position of the cross of isocline) of the flowing solution

must be determined. The methods used for this determination, and the values obtained for the angle of isocline under varying conditions, have been described in the preceding paper.

The angle of isocline being known, the planes of polarization of the crossed nicol prisms must now be set at 45° to the cross of isocline. Before this setting is made, the motor which has been rotating the outer cylinder is stopped, and the solution allowed to come to rest.[6] The connecting rod which has been used for simultaneous rotation of the nicols, is disconnected, and the analyzer set very carefully at 45° to the previous setting which coincided with the arm of the cross of isocline. With the aid of the half-shadow, the polarizer is set at 90° to the analyzer. The quarter wave-length plate is then inserted, and so oriented that the plane-polarized light from the polarizer passes through without change. This setting is very delicate, the smallest deviations being detected by the half-shadow.

The measurement of double refraction can now be made. The motor is switched on, and while the outer cylinder is rotating with a constant velocity, ten to twenty readings of the angle Δ° are taken. The velocity can be altered at will by a system of reducing pulleys, and uniform rotation secured at speeds varying from 30 to 360 R.P.M. At one or two points, sets of readings are taken at a given velocity near the beginning and again near the end of the experiment. If the readings are not reproducible, all the measurements are rejected. The sets of reproducible readings obtained in this manner when plotted graphically will be referred to as Δ-Ω curves. Whenever it was experimentally possible, the effects of other parameters on the double refraction of flow were always studied by means of such Δ-Ω curves. Although in the present investigation this has in many instances not been possible, it will be the aim of future studies to fulfil this important experimental requirement.

Optical System.—The arrangement of lenses and diaphragms has been sketched in the preceding paper. To determine the angle

[6] There is a period (lasting with concentrated solutions about a minute) during which a small amount of double refraction remains in the solution before the anisotropic particles have become completely disoriented, after the shearing forces have ceased to act. Accurate settings for crossing the nicols can only be obtained when this residual effect has disappeared.

of isocline no particular optical refinements are necessary; for
the determination of double refraction of flow, however, certain
conditions must be rigorously fulfilled.

In the first place, all lenses and glass parts (such as the glass
bottoms of the outer cylinder and the water jacket) must be
isotropic. This has been tested and found to be the case.
Secondly, the light beam traversing the annular space between the
concentric cylinders should be strictly parallel. If this were
experimentally possible, each individual pencil of light would pass
through a layer of muscle globulin which from top to bottom has
been subjected to the same shearing stress; for it can be assumed
that this stress is everywhere the same throughout one concentric
layer. Ideally, one should measure the double refraction pro-
duced in one infinitesimal light pencil which has passed through
a strictly concentric layer of the flowing liquid along the axis of
the optical system of the apparatus. If this axis is always the
same distance from the inner cylinder (a condition which can be
very closely approached by the adjustments described at length
in the preceding paper) all the optical measurements should be
strictly reproducible. However, this ideal accuracy could be
attained only by studying one infinitesimal pencil of light. To
produce adequate illumination one must observe by means of a
light beam of finite breadth, and accept the inevitable small
inaccuracy involved.

After some experimentation, the following arrangement was
adopted, which proved satisfactory within the limits of accuracy
desired. The condenser (C_3 in Fig. 4 of the preceding paper),
the objective O, and the ocular Oc were rendered telecentric with
respect to the annular space, by means of three diaphragms with
small openings. Telecentric systems possess the general advan-
tage that the image of any object which is outside of the focus of
the system, is formed only by beams lying symmetrically to an
axis which is always parallel to the optic axis of the system. If
the openings of the diaphragms are made small enough, the ideal
case of an image formed only by incident parallel light is very
closely approached. The diaphragms in our experiments on
double refraction allowed beams which diverged from a strictly
parallel condition by 34', to enter the image-forming system.
Hence almost the whole length of the annular space appeared to be

in focus at once when viewed with the ocular (*Oc*). Therefore each pencil of light passed very nearly through the same concentric layer of fluid on its way through the annular space. It is impossible, however, to make a measurement only on the pencil of light which has passed exactly through the axis of the system (this axis, after the adjustments described in the preceding paper, is indicated by the intersection of the cross hairs in the ocular). The image employed for the setting of the half-shadow corresponded to a circular area, 1.5 mm. in diameter on the bottom of the annular space, and 3.2 mm. on the top (due to the unavoidable divergence of the image-forming beams). However, the double refraction in this area varied so slightly that no serious disturbance of the setting was experienced.

All muscle globulin solutions produce a slight rotation of the plane of polarization (about 1° for a 1 per cent solution). This has been compensated by always setting for crossed nicols while the solution was in the apparatus. The justification for this detail of procedure remains an open question. It is possible that this rotation is not the same for a given solution when it is flowing and when it is at rest. In this case a certain small error would be inherent in all our double refraction measurements.

There is always a small layer of solution which lies below the inner cylinder and is not subjected to the same shearing stresses as the rest of the fluid. When the annular space is full, there is also a small layer of this kind above the inner cylinder and below the glass plate. These disturbing effects were disregarded in all experiments made when the annular space was completely filled. When the filling was only partial, the "bottom effect" could be determined by measuring the double refraction at constant angular velocity as the solution was filled in to different heights in the annular space. In this case the double refraction $\Delta°$, was found to increase as a linear function of the volume of muscle globulin solution added, according to the equation

$$\Delta = C(V - V_0) \tag{4}$$

The "dead space," V_0, was determined by graphical extrapolation and found to be 3 to 5 cc. The disturbing effect due to the dead space at the top when the cylinder is full has not yet been measured. It is also unknown whether the percentage disturbance

due to the dead space is the same for varying values of $\Delta°$, and varying values of temperature, viscosity, and other variables.

A careful examination of these and other methodical inaccuracies awaits further investigation. The effects above discussed appeared to be small, and were neglected in the present studies, to obtain a first approximation to the complex behavior of muscle globulin.

Experiments.

The amount of double refraction produced by a given muscle globulin solution is a function of (1) the depth of the layer of solution traversed by the light beam, (2) the number of anisotropic particles present within that layer, *i.e.* the concentration of protein, (3) the shearing stresses between concentric layers in the solution, determined by the angular velocity of the outer cylinder, the distance between the outer and inner cylinders, and the complicated viscous and elastic forces in the solutions discussed at length in the preceding paper, (4) the disorientation produced by the temperature motion of the molecules, (5) the pH, the nature of the salt in which the protein is dissolved, the ionic strength, and the action of denaturing agents. Possibly other factors, such as the refractive index of the medium, are also involved.

The first factor was in general controlled in these experiments by observing through the same depth of solution throughout an experiment. Generally the annular space between the cylinders was entirely filled, the observations being made through a layer of solution 10 cm. deep, between the upper and lower glass plates. In other cases the annular space was only partially filled, and the volume of solution was kept constant.

In studies of double refraction as a function of concentration, successive dilutions were carried out with the same salt solution in which the protein was dissolved, so that pH and ionic strength were maintained constant throughout (except for the small unknown element of ionic strength due to the protein itself). Except when it was desired to study double refraction directly as a function of temperature, the temperature was kept constant at 1–4° by the thermostat.

Experimentally it is almost impossible to modify one of the important variables in the system without modifying others simul-

taneously. Thus, a change of protein concentration inevitably alters also the viscous and elastic forces in the solution; a rise of temperature alters the viscous and elastic forces and also increases the temperature motion of the molecules. This close interrelation between the variables should be borne in mind in the consideration of the experiments which follow.

I. *Double Refraction as a Function of Angular Velocity, for Different Protein Concentrations, Temperature, pH, and Ionic Strength Being Constant.*—The variation of double refraction with the applied shearing stress is the most fundamental relation requiring investigation. If this relation were accurately known, the double refraction due to orientation of anisotropic particles could be differentiated from the photoelastic effects due to the "rigidity" of the fluid.[7] At present, however, there is no accurate method of measuring the shearing stress in a rigid fluid. The closest approximation at present attainable involves the measurement of double refraction as a function of the angular velocity of the external cylinder. In a fluid of normal viscosity, this measurement would permit the direct calculation of the shearing forces at any point, if the viscosity of the solution and the dimensions of the apparatus were known. The shearing forces would be directly proportional to the angular velocity and could be calculated from Newton's differential equation of flow. In muscle globulin, however, the shearing forces due to viscosity are augmented by forces due to elastic deformation, the latter bearing a still unknown relation to the angular velocity of the outer cylinder.

In Fig. 2 is shown a series of characteristic Δ-Ω curves obtained on cow muscle globulin, C.M.G. XIV, for eight different protein concentrations. Δ was measured at five or six different angular velocities for each concentration, each individual point being the average of ten to twenty readings. The experimental points have been fitted by smooth curves, whose form is typical for all muscle globulin preparations so far examined.

The protein, which had been four times reprecipitated, was dissolved in potassium phosphate buffer, pH 6.8. Successive dilutions were carried out by adding measured volumes of the

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[7] A preliminary attempt to determine this relation directly by transforming the inner cylinder into a Couette-Hatschek viscosimeter, proved unsuccessful.

same buffer to measured amounts of the protein solution.   Concentration is expressed as mg. of protein nitrogen per liter.   The nitrogen in the most concentrated and the most dilute solutions was determined directly by Kjeldahl analysis; intermediate concentrations are calculated from the volumes used in dilution.   The temperature was maintained at 3° throughout.   The apparatus was completely filled each time between the upper and the lower glass plates, the length of the fluid column being therefore always 10 cm.   Inner Cylinder 1 was used (for dimensions, see description
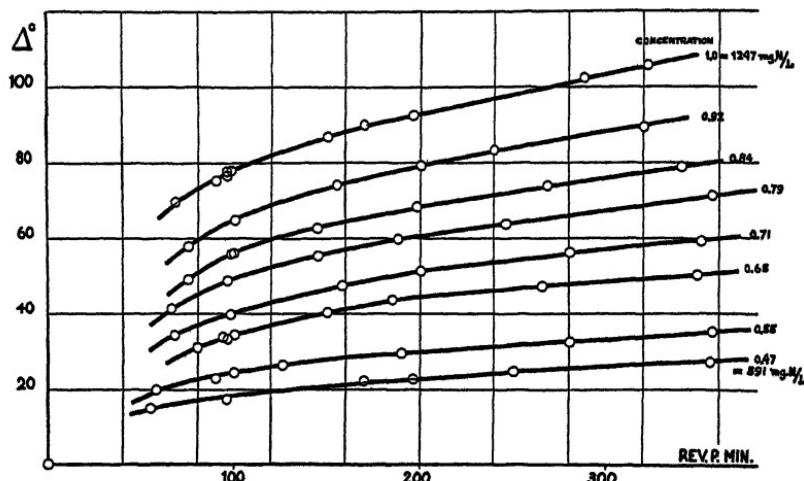
Fig. 2. Characteristic Δ-Ω curves obtained on cow muscle globulin, C.M.G. XIV, for various protein concentrations at different angular velocities for each concentration, each individual point being the average of ten to twenty readings.

of apparatus in preceding paper).   Measurements were always made at the same distance between the inner and outer cylinders, as described in the preceding paper.

Fig. 3 shows a similar set of Δ-Ω curves for rabbit muscle globulin R.M.G. III.   Inner Cylinder 2 was used; the velocity gradient for a given speed of revolution is approximately twice as great as for the data of Fig. 2.

The protein had been twice precipitated by dilution, and was dissolved in potassium phosphate buffer, pH 6.8, ionic strength 0.56.   The method of dilution, the determination of protein

concentration, the temperature, and the length of the fluid column were as described above for Fig. 2.

The character of these curves is notable in two respects. In the first place, there is no suggestion of the ageing effect which has been generally found in the past, in colloids showing double refraction of flow. In the V_2O_5 sol, for example, Freundlich, Stapelfeld, and Zocher (9) found a steady increase with time in the double refraction produced by a given preparation. The
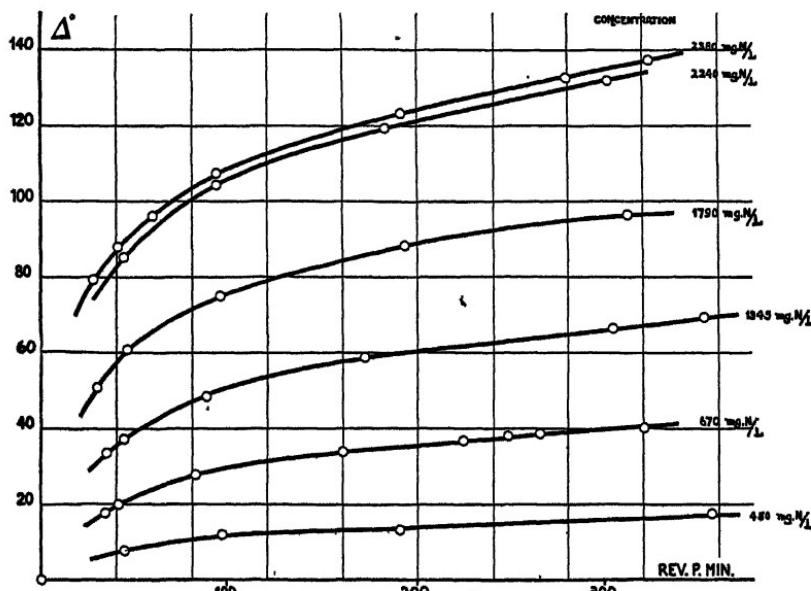
Fig. 3. Δ-Ω curves for rabbit muscle globulin (R.M.G. III) with inner Cylinder 2 and a velocity gradient for a given speed of revolution approximately twice as great as for Fig. 2.

curves which they obtained relating double refraction to velocity of outflow (corresponding roughly to our Δ-Ω curves), changed their shape with the age of the preparation, and assumed more and more the character of saturation curves. Finally with very old preparations a limiting value seemed to be attained, and no further change was observed.

No such effects have been observed in muscle globulin. The amount of double refraction is a function of many variables but

is entirely independent of time (except for denaturation effects, discussed below, which always cause the double refraction to *decrease* with time, never to increase).

In the second place, the shape of the Δ-Ω curves which we have obtained is very characteristic, and has been found in every preparation so far examined. For low angular velocities the curve rises fairly steeply, and is markedly concave to the axis of the abscissa. For higher angular velocities it has a smaller slope and approaches more nearly to a straight line. This characteristic
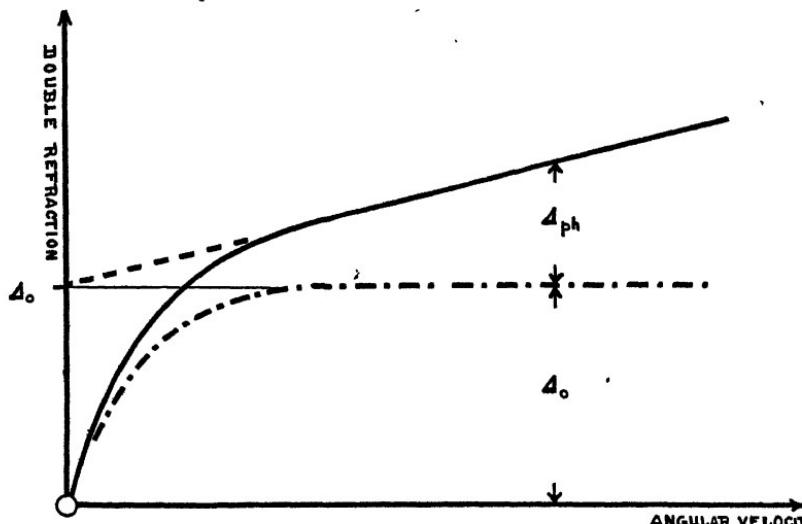
FIG. 4. Curve resulting if a simple saturation curve and a linear term be superimposed. The saturation values Δ_0 are obtained by linear extrapolation.

shape, and the reproducibility of the curves, would indicate that double refraction of flow is a fundamental property of muscle globulin.

No general theory of double refraction for a system like muscle globulin has yet been advanced. Hence no final conclusions can be drawn from the Δ-Ω curves. We present, however, three tentative interpretations of the data we have obtained, which all appear to deserve consideration.

1. If a simple saturation curve and a linear term were superim-

posed (Fig. 4) the resulting curve would be extremely similar to those obtained experimentally.   Beyond the point where saturation for one component of the curve is reached ($\Delta = \Delta_0$) the curve becomes a straight line described by the equation

$$\Delta - \Delta_0 = \Delta_{ph} = k \, \Omega \tag{5}$$

Below Δ_0 the curve slopes down to 0, its character being determined by both the linear and the unsaturated component.   The limiting value for the component which approaches saturation ($\Delta = \Delta_0$) can be determined by extrapolating the upper (linear) portion of the curve to its intersection with the ordinate ($\Omega = 0$). (See Fig. 4.)

These two components may be given a definite physical meaning. The component which approaches saturation with increasing angular velocity would represent the double refraction produced by the orientation of anisotropic molecules, which become completely oriented `above a certain angular velocity.   The linear component (Δ_{ph}) on the other hand might be a photoelastic effect, increasing in direct proportion to the angular velocity, and due to the rigidity of the solution.

This conception offers a simple explanation of the Δ-Ω curves and accords well with the conclusions to which we have been led by the study of the angle of isocline.   It should be pointed out, however, that a very slight curvature still remains in the upper portions of the experimental curves, a fact which does not quite accord with this simple picture.

2. The form of the curves might also be interpreted in terms of two superimposed orientation effects possessing different limiting values.   The asymmetric muscle globulin particles may be regarded as elongated spheroids.   A rotational spheroid would orient its long axis in the direction of largest stress, and this orientation would approach a definite limiting value as the stress increased. A spheroid with three different axes, however, would orient at relatively low stresses with its longest axis in the direction of largest stress.   With increase of the applied stress, a second orientation would occur, involving the smaller axes, and not reaching a limiting value (at which the homologous axes of all the particles would lie parallel) until much larger stresses are applied.   This theory is also in accord with the data presented in Figs. 2 and 3.

This would mean that orientation of the longest axis is complete at the higher velocities shown in those figures, but that the complete orientation of the other axes is very far from being attained, and would require much higher shearing stresses than we have employed.

3. According to another interpretation, the whole curve is a complicated saturation curve in which complete saturation is never reached.   The orientation of muscle globulin particles in a mechanical field would on this theory be analogous to the orientation of dipoles in an electric field (Debye).   This orientation is described by the equation originally developed by Langevin in his study of paramagnetism (see the treatment by Debye (7)), which gives a form of curve quite similar to those obtained for muscle globulin.   Such an assumption, however, would appear to involve a very small size for the muscle globulin particle; for the product of the "moment" of the particle and the "field intensity" of the mechanical forces must be small in comparison with the temperature energy kT.   In view of the large molecular weights of the proteins in general, this suggestion appears improbable.

At present we are not in a position to decide in favor of any of these three theories, and must await further theoretical developments.

It is therefore at present impossible to establish a definite relation between double refraction and concentration, since concentration enters as an unknown parameter into the Δ-Ω relation.   Therefore no calculation of the anisotropy of the muscle globulin particle can be made.   While this anisotropy has been qualitatively established it remains quantitatively unknown.

To obtain some light on the problem, however, explanation (1) above has been accepted as a working hypothesis.   The saturation values Δ_0 have been obtained from Figs. 2 and 3, by linear extrapolation, as described above and illustrated in Fig. 4.   The saturation values are plotted as a function of concentration in Fig. 5.

The saturation values Δ_0 appear to bear a nearly linear relation to the concentration, especially in the upper part of the curve.   The results obtained on the two preparations cannot be compared directly, since the measurements were made with two different inner cylinders.   The functional relation, however, appears to be nearly the same in both cases.

II. Double Refraction as a Function of Temperature. Protein Concentration, pH, and Ionic Strength Constant.—The tempera-
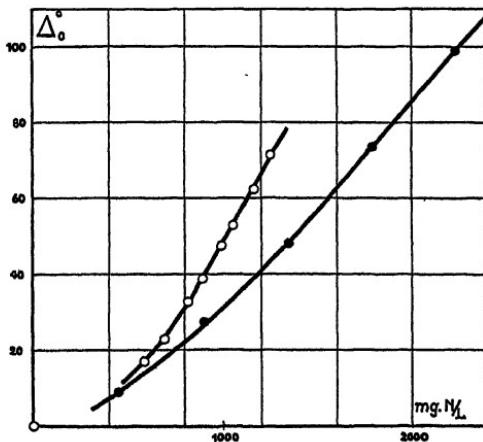
FIG. 5. Saturation values Δ°_0 plotted as a function of concentration. Data for cow muscle globulin, C.M.G. XIV, indicated by O, and rabbit muscle globulin, R.M.G. III, by ●.
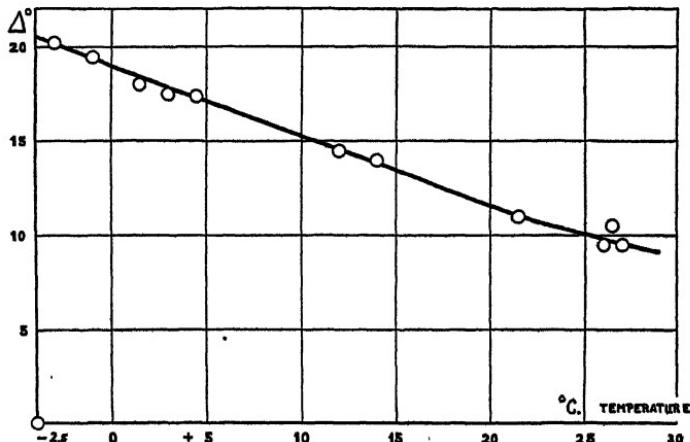
FIG. 6. Variation of double refraction for cow muscle globulin, C.M.G. XI, with varying temperatures, other factors being constant.

ture of the protein solution may be varied by varying the temperature of the large thermostat bath from which water is pumped and circulated around the cylinders. Fig. 6 shows the variation of

double refraction with temperature for cow muscle globulin, C.M.G. XI, between −2.5° and 27°. The speed of the outer cylinder was constant at 96 R.P.M. throughout the experiment.

The muscle globulin was dissolved in sodium chloride, pH 7. The concentration was 592 mg. of protein nitrogen per liter. Angle of isocline 64.5°.

At the highest and the lowest temperatures studied, conditions could be kept constant, and constant readings for double refraction obtained. For technical reasons, the intermediate temperatures could not be kept very constant. The experiment was begun at 1.5° where the first measurements were made. The bath was then slowly warmed to 27°, readings being taken at numerous intermediate points. Temperature was kept constant at 27° until readings of Δ were constant; the bath was then slowly cooled to −2.5°, readings again being taken throughout the time that the temperature fell; after equilibrium had been reached, it was brought up again to 27°, and thence down to 2.9°. As the temperature was altered, the temperature of the muscle globulin solution rose or fell with that of the water bath outside, but with a certain lag. Consequently simultaneous readings of the double refraction in the solution and of the temperature of the water bath outside are not strictly comparable. The lag was compensated by taking, for each temperature measured, the average of two double refraction readings, one with rising, the other with falling temperature. The points plotted in Fig. 6 represent such averaged values, except at the upper and lower temperature limits, where constant equilibrium values were obtained.

Temperature has probably two effects on the orientation of the anisotropic particles. Increasing temperature diminishes the viscosity and therefore the shearing forces which produce orientation, and also increases the thermal agitation of the molecules. Both these effects decrease the amount of double refraction produced at a given angular velocity.

To throw more light on the influence of temperature, Δ-Ω curves were determined for cow muscle globulin preparation C.M.G. X-3 at two different temperatures (Fig. 7).

Increase in temperature by 20° produces a very marked change in double refraction. The Δ-Ω curve is lowered, much as if the protein concentration had been diminished. This is probably

chiefly due to the change in viscosity. The slope of the linear portion of the curve appears to be somewhat smaller at the higher temperature. If explanation (1), given above, be accepted, this would imply a diminished photoelastic effect. The saturation point, however, has not shifted markedly.
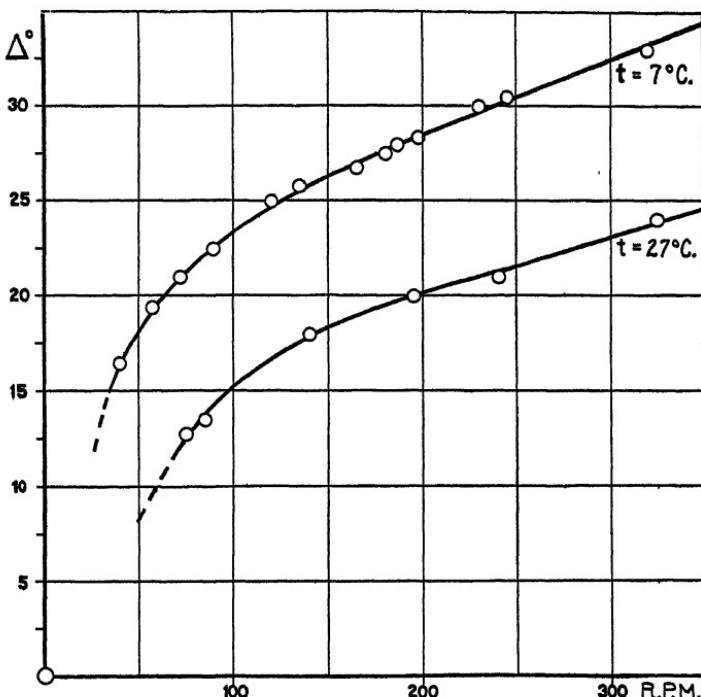
Fig. 7. Δ-Ω curves for cow muscle globulin, C.M.G. X-3 (concentration 633 mg. of protein nitrogen per liter, pH 7, angle of isocline 65°) at two different temperatures.

The experiments on the temperature effect did not always give such reproducible results. Not infrequently the value of Δ was permanently reduced after the solution had been maintained for some time at 25–30°. This we ascribe to partial denaturation of the protein at the higher temperature, for it is shown in what follows that denaturation is accompanied by loss of double refraction. In many experiments, however, no such change has been observed; temperature could be raised or lowered several

times, without appreciably altering the value of Δ obtained at any given temperature. This was true of the experiments represented in Figs. 6 and 7; the changes represented there are strictly reversible; *i.e.*, they are simple temperature effects uncomplicated by denaturation.

III. Influence of Viscosity on Double Refraction of Flow.—The old "elastic" theories explained double refraction of flow as a pure photoelastic effect, due to the shearing stress within the fluid. On the basis of these theories, the viscosity of the liquid would play a primary rôle; the double refraction in flowing fluids should increase with their viscosity, and reach maximal values in extremely viscous fluids. We have examined glycerol, which is about 800 times as viscous as water at room temperature, placed between the concentric cylinders, the outer cylinder being rotated with increasing speed up to 1500 R.P.M. No trace of double refraction could be detected.[8] On the other hand, a muscle globulin solution which had nearly the outflow time of water in a capillary viscosimeter, gave a double refraction $\Delta = 11°$ at a speed of 96 R.P.M.

Clearly, therefore, viscosity is not the main agency effective in producing double refraction of flow. Orientation of anisotropic particles is in all probability the primary underlying factor. Viscosity is however a sufficiently important factor to deserve careful study. The shearing forces which bring about orientation are a function of the viscosity, and of the angular velocity of the rotating cylinder. Different measurements of double refraction are strictly comparable only if made at the same viscosity and angular velocity. While a constant angular velocity is readily maintained, the viscosity is altered if any one of the other parameters—such as concentration, temperature, pH, ionic strength—is varied. Different measurements could be reduced to a comparable basis if the relation between viscosity and double refraction were known. For a pure liquid, according to the theory of Raman and Krishnan (23) this relation should be linear, and the calculation is simple. For a solution like muscle globulin, however, the relation is unknown, and is in any case complicated by the elastic forces present which cause the apparent viscosity to vary with the applied shearing stress.

[8] Vorländer and Walter (31) detected a very slight amount of double refraction in glycerol, using a very delicate detecting instrument, and shearing stresses much greater than any we have employed.

To give a general indication of the relations involved, however, measurements of double refraction were made as the viscosity was varied, the total quantity of protein present being the same throughout the measurements. The results, for cow muscle globulin, C.M.G. XXII, and rabbit muscle globulin, R.M.G. III, are shown in Fig. 8, Δ being plotted for each solution against its outflow time θ' in an Ostwald viscosimeter relative to that of distilled water (θ_{H_2O}).
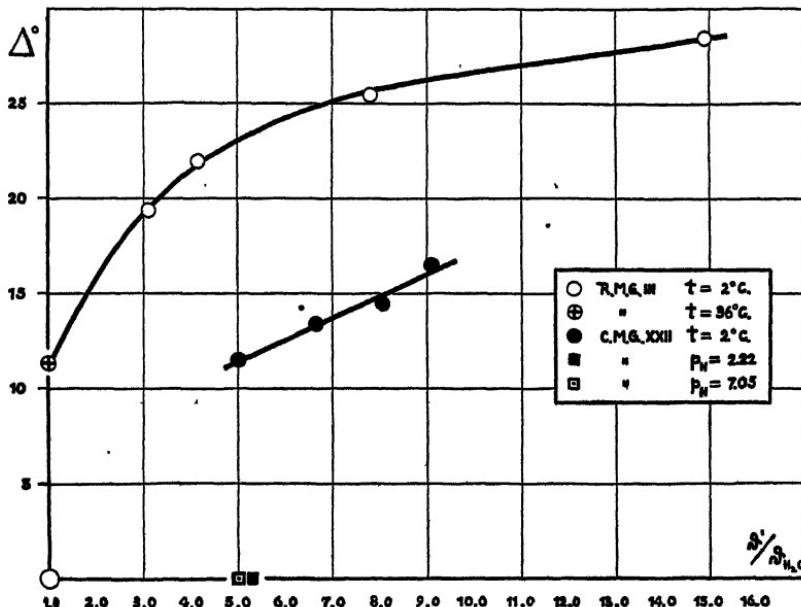
Fig. 8. Double refraction measurements made with varied viscosity for cow muscle globulin, C.M.G. XXII, and rabbit muscle globulin, R.M.G. III.

Rabbit Muscle Globulin, R.M.G. III.—20 cc. of twice precipitated protein were taken (2240 mg. of protein nitrogen per liter) and placed in the annular space between the cylinders (inner Cylinder 2 being used). Angle of isocline 78°. The value of Δ was determined, and the solution was then diluted with the same potassium phosphate buffer (ionic strength 0.56, pH 6.8) in which it was dissolved. In diluting, the buffer was run directly into the annular space, and the apparatus rotated until complete

mixing had occurred.   The new value of Δ was then read.   Thus, although concentration and viscosity were altered, the total amount of protein contributing to the value of Δ remained essentially constant throughout the experiment.[9]   The rotation of the outer cylinder was constant at 96 R.P.M.   The extreme left hand reading (Fig. 8) was taken by warming the solution up to 36°.   Simultaneous readings of outflow time in Ostwald viscosimeters were taken with another portion of the original muscle globulin solution, which was successively diluted with buffer in the same proportions as the solution in the cylinder.   The outflow time of the solution at 36° is compared with the outflow time of water in the same viscosimeter at 2° (as for the other measurements), as what was desired was an approximation to the *absolute* values of the shearing stress at different temperatures, not the relative values at each temperature.

Cow Muscle Globulin, C.M.G. XXII.—This (805 mg. of protein nitrogen per liter, angle of isocline 66.5°) was studied in a similar manner.   It was dissolved in potassium phosphate buffer, ionic strength 1.25, pH 6.8.

Although the curves are unsatisfactory and represent only preliminary measurements, certain facts stand out.   In the first place, the values of Δ decrease with decreasing viscosity, but much less rapidly than the viscosity decreases.   While the apparent viscosity was varied 16-fold in the study of the rabbit preparation shown in Fig. 8, the values for double refraction varied less than 3-fold.   Even at very low viscosities in muscle globulin preparations, there is sufficient orientation to produce well marked double refraction at very moderate angular velocities.   This suggests that the muscle globulin particles must be large and strongly asymmetric, since otherwise the very moderate shearing forces employed would never suffice to bring about orientation.

Not only may solutions of very low viscosity show well marked double refraction, but solutions with much higher viscosities may show no double refraction at all.   The two points lying on the axis of the abscissa should be noted in this connection.   They were obtained on cow muscle globulin, C.M.G. XXII (the same preparation shown above), which had been brought by the addition of

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[9] It was not absolutely constant, because of the presence of the "dead space" below the inner cylinder.

phosphoric acid to pH 2.22, and then by the addition of alkaline phosphate back to pH 7.05. (pH values were determined by the hydrogen electrode.) The double refraction has completely disappeared in the acid solution, and is not restored by bringing the protein back to neutrality, although the viscosity is as great as that of the preparation when it gave 11° of double refraction before treatment with acid.

This leads to another subject—the loss of double refraction through the action of denaturing agents.

IV. Denaturation of Protein Is Accompanied by the Disappearance of Double Refraction of Flow.—A given muscle globulin
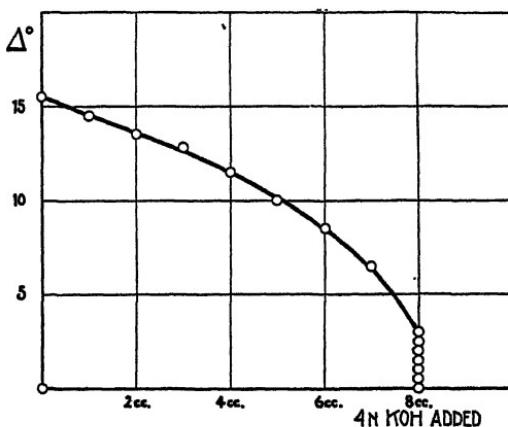
FIG. 9. Curve showing the loss of double refraction produced by the addition of alkali.   Final pH 10.3.

preparation, kept in the cold, free from bacteria, and dissolved in chloride or phosphate solution, at pH 6.5 to 7.5, exhibits a value for double refraction of flow which is practically independent of time.   A very slow diminution may be observed, due presumably to spontaneous denaturation of the protein, which goes on to some extent even at low temperatures.   In the course of a week, however, the change is scarcely appreciable.

The double refraction is, however, rapidly destroyed by a number of agencies.   The effect of alkali is clearly shown in Fig. 9, which represents an early and rather crude experiment, but brings out clearly several of the factors involved.

Very strong alkali had to be used in this experiment, as the protein was in a heavily buffered phosphate solution at pH 7. It will be noted that each addition of alkali produced a definite drop in the double refraction, but the value of Δ came in each case to a constant level when mixing was complete. When 8 cc. of alkali had been added, however, no constant value was obtained but the value of Δ dropped rapidly to zero in the course of about 10 minutes (indicated by the succession of descending points bunched together). The drop in the values of Δ due to the earlier additions of alkali cannot be attributed primarily to dilution, for the volume of alkali added was very small, relative to that of the protein solution; it is probably due to a rapid momentary denaturing action on the protein of the added alkali, before it has become mixed with the buffer present. The final pH of the solution was 10.3.

In other experiments, the protein was dissolved in an unbuffered solution such as NaCl, and dilute alkali (0.1 N to 0.01 N) slowly added. Little change in double refraction was observed until a critical point was reached, at which the double refraction disappeared completely in 10 to 20 minutes.

When the point had been reached at which the double refraction had completely disappeared, a portion of the solution was removed, and its pH determined on the hydrogen electrode. This pH value lay invariably between 10.3 and 11.2, generally about 10.5.

In a later experiment, equal portions of rabbit muscle globulin, R.M.G. IV, were taken and dissolved in borate buffers of varying pH. (The pH of the resulting solution was always measured directly on the hydrogen electrode, as the buffering power of the protein present altered the pH markedly.) Such a solution at pH 9 was run between the concentric cylinders for 18 hours at 4°, and no measurable change in Δ was detected during this time. Similarly at pH 10, no change was observed in an experiment which lasted 8 hours, and was then discontinued. At pH 11.2, however, a rapid alteration took place. About two-thirds of the original double refraction had disappeared within the first 10 minutes, and there was no detectable trace left at the end of 30 minutes (temperature 4°).

The addition of acids produces a similar destructive effect, although the exact pH zone in which the change begins has not yet been so clearly defined. Muscle globulin dissolved in acetate

buffer at pH 4.6 still shows well marked double refraction of flow, while at pH 2.2 the double refraction has entirely disappeared. We have not yet sufficient data to state exactly where the change occurs.

In no instance in our experiments has double refraction been restored after its loss in acid or alkaline solution, by bringing the pH back to the neutral range. It may be that under suitable conditions the change is reversible, but under the conditions of our experiments it has not been reversed.

All these observations apply both to cow and rabbit muscle globulin.

It should be noted that muscle globulin solutions, altered in this manner by acid or alkali, are still clear solutions. They are soluble in the neutral range in the presence of salt, can be precipitated and then redissolved like the original protein by increasing and decreasing the ionic strength, precipitated by small amounts of acid (at pH 5 to 6) and redissolved by excess of acid. In all these respects their behavior is (qualitatively at least) so like that of the original protein that the casual observer might well suppose that no change had occurred. The measurements on double refraction, however, testify unmistakably that a profound alteration has taken place.

These results are closely parallel to those obtained on other proteins by quite different methods. It has been recognized by many investigators that proteins are altered in their properties in mildly acid or alkaline solutions, and the observations made by Svedberg (29) and his colleagues with the ultracentrifuge have brilliantly confirmed this fact. They have found each pure protein to be monodisperse only within a definite pH range, whose exact limits are characteristic for the particular protein involved. Serum albumin, for example, is stable between pH 3.6 and 9; serum globulin between 4 and 8.5 (Svedberg (29)). In more acid or more alkaline solutions, the protein ceases to be monodisperse, breaking down into smaller particles, or forming aggregates of varying size. The electrical and optical properties of these various proteins, however, alter very little until breakdown into particles smaller than the egg albumin molecule occurs (a change which generally involves more drastic chemical treatment). Muscle globulin, to judge from the study of its double refraction of flow,

possesses a similar pH zone within which it is stable, and in this instance also the altered protein is very similar to the original material in many of its properties.

All typical denaturing agents which we have tested produce a similar destruction of the double refraction. Concentrated solutions of urea, for instance, readily dissolve muscle globulin. On diluting the resulting solution, the protein precipitates and cannot be redissolved by neutral salts. Similar observations on other proteins have been made by Anson and Mirsky (2) and by Burk and Greenberg (5). If muscle globulin dissolved in urea is examined between the rotating concentric cylinders, no trace of double refraction can be detected, even 5 minutes after the urea has been added. 8 per cent solutions of potassium iodide and potassium thiocyanate also produce complete loss of double refraction within half an hour at 4°, without precipitating the protein. The destructive influence of heat has been discussed in a previous section. According to Anson and Mirsky (2) all these agencies produce typical denaturation of hemoglobin and egg albumin. It may therefore be stated that *denaturation of muscle globulin is accompanied by the loss of its characteristic property of producing double refraction of flow.* This fact affords further justification for the belief that the double refraction is intimately related to the chemical structure of the protein molecule.

It seems probable from some of our observations (although we cannot yet be positive) that a similar destructive effect is produced by allowing the protein to stand several hours in the precipitated condition, especially if the temperature is not kept low. Under these conditions the protein passes slowly into a permanently insoluble form. Probably less radical alterations (which are still sufficient to destroy the double refraction) occur before the stage of complete insolubility is reached. At all events, these observations serve to emphasize the need for great care in the extraction and purification of the protein. They also explain why strong acids or alkalies are not allowed to come in contact with the protein even for a moment during the preparation.

V. Studies on the Muscles of Other Animals.—Besides the cow and the rabbit, we have observed double refraction of flow in the muscle globulin of the frog, and in protein extracted from the foot muscle of the marine snail, *Busycon*. The frog globulin was pre-

pared from the fresh chopped hind leg muscle, in a similar manner to that described (8) for the cow and rabbit. The frogs were not perfused. After the extract had been once precipitated by diluting with water, centrifuged, and redissolved in potassium phosphate, a dilute solution was tested and found to give $\Delta = 12°$ at 96 R.P.M. The experiment was not carried further.

For the snail muscle we are indebted to Dr. A. C. Redfield, who had previously bled the snails and removed the hemocyanin. On extraction of the ground muscle with potassium phosphate (ionic strength 1.3, pH 6.8) and subsequent filtration through filter paper pulp, a liquid with high light-scattering power was obtained. This gave 7° of double refraction at 96 R.P.M. (inner Cylinder 1). The angle of isocline was 70° (the cross of isocline was rather indistinct). On the addition of ammonium sulfate (adjusted with NaOH to pH 7) to the filtered extract, a heavy precipitate resulted when the solution was one-third saturated with ammonium sulfate (phosphate of course was also present). This precipitate, on being centrifuged, yielded a solution on the addition of water which gave 17° of double refraction under the same conditions.

These very crude preliminary observations are of some interest as showing that doubly refractive protein may be obtained from the smooth muscles of an invertebrate as well as from the striated muscles of higher animals. It is hoped to extend these studies in future to other types of animals, and to the proteins of other doubly refractive tissues (such as the mucoproteins of tendon).[10]

VI. *The Gans Effect and Its Relation to the Asymmetry of Muscle Globulin Particles.*—The studies which have been reported afford evidence that the muscle globulin particle is asymmetric. Without this assumption it appears difficult to account for the double refraction of flow. There exists, however, an entirely independent method of testing this assumption, which was developed by Gans (11). He showed the degree of depolarization of the scattered light from a solution to be dependent on the asymmetry of the particles (or molecules) present. The scattered light is measured at an angle of 90° to the incident plane-polarized beam. Lange

[10] None of the blood proteins (with the possible exception of serum globulin) shows double refraction of flow, even at high concentrations. No trace of double refraction was observed in a casein solution containing 4.35 per cent of protein; and egg albumin also is negative.

(17) has measured the asymmetry of the particles in various colloidal solutions by this method. Preliminary measurements of this effect have been made on muscle globulin solutions, with a König-Martens spectrophotometer and an optical arrangement very similar to that employed by Lange. The degree of depolarization, which was measured and calculated according to the formulas used by Lange, indicated the presence of markedly asymmetrical particles in the solution. The degree of asymmetry corresponded roughly to that found in V_2O_5 sols of intermediate age.

Discussion of Experimental Data.

The study of the angle of isocline has led us to the belief, expressed in the preceding paper, that the particles responsible for the double refraction are of constant size and shape. The data presented in this paper would appear to strengthen this view. The Δ-Ω curves are always of the same characteristic shape from one preparation to another; and for any given preparation, the curves are independent of time and reproducible from one day (or week) to the next (except for slow denaturation). This behavior contrasts markedly with that of the V_2O_5 sol (9), in which the curves change profoundly with time for any given preparation. This ageing effect in V_2O_5 sols is ascribed largely to the steady increase in length of the needle-shaped V_2O_5 particles, a view which is confirmed by x-ray studies and by other methods. Hand in hand with this alteration in size go changes in the viscous and elastic properties of the sols, which alter the shearing forces to which the particles are subjected. The effect of age on the double refraction in V_2O_5 sols is thus highly complicated. No indication of any similar effects has been obtained with muscle globulin, from which it may be inferred that the size and shape of the anisotropic muscle globulin particles does not change with time.

Although the *form* of the Δ-Ω curves is characteristic for all preparations thus far studied, different preparations do not in general give quantitatively identical curves. Two chemically identical preparations should—when dissolved in the same salt, at the same protein concentration, at the same temperature, pH, and ionic strength—give identical Δ-Ω curves in the same apparatus. Such strictly comparable preparations have not yet been

obtained. For different preparations of the same nitrogen content (as determined by Kjeldahl analysis) different curves have been obtained. It might be supposed that we are dealing with a mixture of doubly refractive proteins, of varying degree of anisotropy, which are extracted in varying proportions in the different preparations. We do not believe, however, that this explanation holds. If a mixture of particles of different size and shape were contributing to the double refraction of flow, different preparations should give very variable angles of isocline, which is not the case. The limiting value of 78° for the angle of isocline, which we have always obtained when a preparation is sufficiently purified, points to a certain uniformity in all the preparations from both cow and rabbit. If the average size and shape of the anisotropic particles varied from one preparation to another, the *form* of the Δ-Ω curves, as well as their dimensions, should vary. Furthermore, all the denaturing agents whose action we have studied destroy completely the double refraction of flow. It appears that only the native protein, and not any of the alteration products,
· contributes to the observed effects.

In our opinion, the observations are best explained by the view that the double refraction of flow is due to one chemical individual —a characteristic protein whose anisotropic particles are uniform in size and shape (monodisperse). This active protein is, however, contaminated by others which are inactive in this respect. These may either be foreign proteins (blood proteins or myogen), or alteration products of the anisotropic protein formed during extraction and purification. The latter alternative seems the more probable, for myogen and the blood proteins differ sharply in their solubility from muscle globulin, and should be quantitatively separated by the process of purification employed (see Paper II of this series (8)). The inactive alteration products of muscle globulin (such as those produced by too acid or alkaline pH) on the other hand, are similar in solubility to the original protein and are probably much more difficult to separate. We believe however that in time these difficulties will be overcome, and the anisotropic material prepared in a pure form, identical in its properties from one preparation to another.

Svedberg and Heyroth (see Svedberg (29)) have studied muscle globulin in the ultracentrifuge and found it polydisperse. This

finding is in accord with the fluctuations in our experiments, discussed above. If our interpretation is correct, however, the anisotropic material is monodisperse, and if adequately purified should so appear in the ultracentrifuge.

We have used the name "muscle globulin" to describe what is almost certainly a mixture of different substances in varying proportions. We propose that the classical name myosin be reserved for what is probably a definite chemical entity—the anisotropic protein responsible for the double refraction of flow. The existence of myosin as a chemical individual is still hypothetical, but the hypothesis is in accord with our own experimental data, and with the great mass of evidence accumulated in recent years, that with sufficient care proteins can be prepared as pure chemical individuals. With the advance of experimental technique, the hypothesis should soon be susceptible of direct proof or disproof.

If myosin is a monodisperse chemical individual, it would appear reasonable to speak of the anisotropic particles as molecules, in the same sense in which one may speak of the hemoglobin or · hemocyanin molecule, for myosin would then satisfy all the criteria for molecular dispersity which have been applied to other proteins. In the absence of final evidence, however, we are content to refer to the anisotropic units as particles.

All the data presented in this and the preceding paper appear to indicate that the myosin particle is anisotropic and asymmetric. The general similarity of muscle globulin to the V_2O_5 sol, for which the anisotropy of the flowing particle has been conclusively shown points to this conclusion.[11] The characteristic shape of the Δ-Ω curves, which contain at least a component which reaches saturation, also would indicate an effect due to the orientation of asymmetric particles. Furthermore the Gans effect gives independent evidence that the particles are asymmetric.

We may now pass to a discussion of the possible functional

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[11] V_2O_5 sols show a definite x-ray diffraction pattern when examined during flow. Dr. R. W. G. Wyckoff, at the Rockefeller Institute for Medical Research, has made x-ray studies on a flowing muscle globulin solution prepared by us, with negative results. Dr. Wyckoff states, however, that these experiments are only preliminary, and that no definite conclusions as to the nature of the muscle globulin solution can be drawn from them.

significance of myosin.   Its characteristic double refraction would
indicate that it is located in the anisotropic disc.   It may be noted
that the same claim was made for myosin in 1881 by Schipiloff and
Danilewsky (25).   They found that a drop of myosin allowed to
dry on a glass slide showed double refraction under the polarizing
microscope as it dried out. Other proteins did not give this effect.
On extracting myosin from the muscle as thoroughly as possible
with ammonium chloride, they observed that very little double
refraction was left in the tissue.[12]   They apparently did not con-
trol for possible double refraction due to salt crystals formed on
drying, and their results were not confirmed by later observers
(von Fürth (10) p. 381). . It is not improbable, however, that they
were observing a genuine effect, due to combined orientation and
photoelastic effects, similar in principle to those producing double
refraction in our experiments.

Pischinger (22) has studied the isoelectric point of the muscle
proteins *in situ* by staining muscle fibers (fixed in alcohol) with
acid and basic dyes at varying pH. He infers an isoelectric
point at pH 4.7 to 4.9 for the protein of the isotropic band, and
at pH 6.2 for the anisotropic band. The latter finding agrees
well with the acid- and base-binding experiments of Salter (24) and
Edsall (8).   However, if taken in connection with the experiments
of Weber (32) on isoelectric points this would lead to the conclusion
that myogen, not myosin, is the protein of the anisotropic band.
This seems an unlikely conclusion.   It is possible that Pischinger
may have seriously altered the muscle proteins by alcohol fixation;
so that at present no final interpretation can be given to his work.

Stuebel's experiments indicate that the double refraction of
intact muscle fiber is largely due to the presence of oriented rod-
shaped particles, small compared with the wave-length of light.
Our experiments give reason to believe that the myosin particle
is anisotropic. The possibility therefore arises that the myosin
particle is the rod-shaped element in the muscle fiber whose
existence is to be inferred from Stuebel's data. On this hypothesis,

[12] They stated that there was some residual double refraction which
could be removed by lipoidal solvents, and was apparently due to lecithin
or some allied substance.   This harmonizes well with Stuebel's observa-
tions.   Unfortunately Schipiloff and Danilewsky did not state whether
the double refraction was positive or negative.

the myosin particles in the muscle fiber represent the Disdiaklasten whose existence was originally postulated by Brücke.

The physical properties of muscle globulin suggest that it may play a part in the "viscous-elastic" effects in muscle, so thoroughly investigated in recent years by Hill and his colleagues (Gasser and Hill (13), Hill (15), and Levin and Wyman (18)). They have ascribed the observed changes in the viscosity of muscle, which occur in contraction, to the formation of a new configuration of the underlying structural elements in the fiber. It was shown by von Ebner that double refraction in the intact muscle fiber decreases during contraction, and Stuebel and Liang (28) have shown that the same is true for rigor produced in various ways. This effect might be interpreted as due to dis- or reorientation of the anisotropic myosin particles. The very high viscosity of muscle globulin solutions, and the elastic forces present in them, make this protein appear particularly fit to function in such a machine as muscle.

Garner (12) and Clark (6) have suggested a liquid crystal structure for the anisotropic disc, and based theories of contraction on this hypothesis. In muscle globulin solutions, we have observed no spontaneous tendency for the particles to orient themselves and form anything analogous to anisotropic liquid crystals. Orientation appears only when shearing stress is applied, and disorientation follows rapidly when the stress is removed. However, we have always worked with very dilute solutions (less than 2 per cent of protein) while the concentration of protein in the anisotropic band may be ten times as high. With such relatively close packing of the protein molecules, and in the presence of lipoids and other substances which perhaps bear a very intimate relation to the proteins, orienting forces may well arise within the muscle fiber, of which we have obtained no hint under the conditions of our experiments.

SUMMARY.

1. Quantitative measurements have been made on double refraction of flow in muscle globulin, with the aid of a Sénarmont

compensator. The values obtained are accurate within 1 per cent for large values of double refraction and somewhat less accurate for smaller values. Double refraction (Δ) and angular velocity (Ω) are found to be related by characteristic curves, which vary with protein concentration. These Δ-Ω curves are reproducible, for any given preparation, over a protracted period of time. There is no suggestion of the ageing effect observed with many doubly refractive colloids, such as the V_2O_5 sol. The general form of the Δ-Ω curves is the same for all preparations examined.

These facts suggest that the double refraction is intimately related to the chemical nature of the protein solution. It is found, in accordance with this view, that typical denaturing agents produce rapid and complete destruction of the double refraction of flow. It would appear that only the undenatured protein is responsible for the double refraction.

The double refraction, is ascribed primarily to the orientation of anisotropic protein particles, due to the shearing stresses which arise during flow. In future it may be possible to relate the double refraction quantitatively to the size, shape, and optical properties of the protein particle. Neither experiment nor theory is yet sufficiently advanced for the accomplishment of this task. Three hypotheses are suggested, however, as possible interpretations of the observed facts, and some tentative conclusions have been drawn on the basis of one of them. The underlying assumption, that the protein particle is anisotropic, receives independent support from studies of the Gans effect (depolarization of scattered light).

2. The evidence appears to us to favor the view that the anisotropic protein particles are of uniform size and shape (monodisperse). The preparations obtained, however, are not completely free of other proteins which do not show double refraction. It is suggested that the name myosin be restricted to the anisotropic protein responsible for the double refraction of flow.

3. Stuebel's work points to the existence of oriented rod-shaped particles, small compared with the wave-length of light, which are largely responsible for the double refraction in the intact muscle fiber. The properties of myosin solutions suggest that they may contain these rod-shaped particles. In any case the double refraction of myosin would indicate its probable location in the

anisotropic disc of the cross-striated muscle fiber, and its general
physicochemical properties suggest that it may play a part in the
functional activity of the muscle.

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THE MOLECULAR SIZE OF THE TYPE III SPECIFIC POLYSACCHARIDE OF PNEUMOCOCCUS.

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Northrop and Anson (1) have recently perfected a method for the estimation of the molecular size of substances in solution. Through the elimination of experimental difficulties which usually accompany the classical methods for determining diffusion coefficients, they have developed a technique whereby the estimation of particle sizes can be accomplished with the greatest facility and simplicity.

It has been our desire for some time to learn the size of the molecules of certain immunologically important polysaccharides. The following is an account of the determination of the molecular weight of the type-specific carbohydrate of Pneumococcus Type III, together with experiments on the size of molecules of hemoglobin, and the polysaccharide of ovomucoid. The first of the latter two determinations was undertaken for the sake of learning the technique and for confirming the findings of Northrop and Anson, and the second was done for the sake of checking the analytical method which we have used to estimate minute quantities of carbohydrate. The reader is referred to the article of Northrop and Anson for a theoretical discussion of this method, as well as for an account of the construction of the apparatus.

EXPERIMENTAL.

I. Standardization of Diffusion Membranes.

1. *Construction of Diffusion Cells.*—The apparatus was essentially the same as that used by Northrop and Anson (1) except that the cells were constructed so as to contain approximately 45 cc. The porous discs used were Jena No. 4 and Alundum R. A. 225, 40 mm. in diameter and approximately 1 mm. thick.

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The whole apparatus was placed in a water bath maintained at 7° (±0.20°) in a constant temperature refrigerating room.

2. Standardization of Diffusion Membranes.—The membrane constant, K, which is the ratio of the thickness of the membrane

TABLE I.

Determination of Cell Membrane Constants at 7°.

Membrane.	Solution.	Time.	Equiva-lent cc. diffused.	Q cc. per day.	Average Q cc. per day.	K cell.
		days	*Q cc.*			
Jena glass No. 1.	Maltose, 10 per cent.*	0.651	1.235	1.896		
		0.214	1.400	1.869		
		0.150	1.282	1.880		
		0.145	1.265	1.828	1.868	0.127
	HCl, 0.1 N.†	0.0410	0.554	13.37		
		0.0416	0.556	13.36		
		0.0451	0.608	13.47	13.40	0.140
Constant of Jena glass cell						0.133
Alundum No. 2.	Maltose, 10 per cent.	0.782	0.1115	1.425		
		0.285	0.414	1.452		
		0.228	0.357	1.561		
		0.652	0.942	1.445	1.471	0.162
	HCl, 0.1 N.	0.1735	1.950	11.20		
		0.0417	1.470	11.27		
		0.0472	1.530	11.23		
		0.0500	1.567	11.34	11.26	0.168
Constant of alundum cell....................................						0.165

* D = diffusion coefficient 10 per cent maltose at 7° = 0.238 cm.² per day extrapolated from Ohölm's data.

† D = diffusion coefficient 0.1 N·HCl [at 7° = 1.88 cm.³ per day extrapolated from Ohölm's data.

to its effective area, was calculated from data obtained by allowing solutions of HCl and maltose (the diffusion coefficients of which are known and which were extrapolated from Ohölm's (2) data) to diffuse through each membrane for a given length of time at a

constant temperature of 7°.   The membrane constant, K, may then be calculated by substitution in the formula

$$K = \frac{D\,t}{Q\,cc.}$$

where Q cc. is the number of cc. of the concentrated solution that contains the amount diffused in the time t, expressed in days, $D =$ known diffusion coefficient of the substance in solution.   The results of the standardization of the cell membranes are given in Table I.

TABLE II.

Diffusion Coefficient of 2 Per Cent CO Hemoglobin at pH 6.81 and at 7°.

Membrane.	Time.	K cell.	Hemoglobin diffused expressed as cc. concentrated solution.	D cm.² per day.	Average D cm.² per day.
	days		*Q cc.*		
Jena glass No. 1.	0.982	0.133	0.341	0.0462	
	0.944		0.308	0.0434	
	1.015		0.360	0.0472	
	0.992		0.338	0.0454	0.0455
Alundum No. 2.	0.970	0.165	0.269	0.0458	
	1.055		0.292	0.0456	
	0.995		0.279	0.0463	0.0459
Diffusion coefficient CO hemoglobin at 7°......................					0.0457

II. Determination of Molecular Size of Hemoglobin.

1. Preparation of Hemoglobin.—Hemoglobin was prepared from horse blood according to the directions of Anson and Mirsky (3).

2. Determination of Diffusion Coefficient of Hemoglobin.—A 4 per cent solution of hemoglobin in water was diluted with an equal volume of 0.1 M phosphate buffer at a pH of 6.81.   The solution was saturated with carbon monoxide and then cooled to 7°.   The cells were filled and were then placed at constant temperature in 20 cc. of 0.05 M phosphate buffer (pH 6.81) which had previously been saturated with carbon monoxide.   The hemoglobin was allowed to diffuse for approximately 24 hours.   The solvent, con-

taining the diffusate, was quantitatively removed and its protein content was determined by micro-Kjeldahl analysis. The results of two diffusion experiments are given in Table II. In this table Q cc. is the amount of carbon monoxide hemoglobin, expressed in terms of the original solution, which had diffused in the indicated time interval. The average value of the diffusion coefficient of CO hemoglobin at 7° is 0.0457 cm.^{2} per day.

3. Calculation of Radius and Molecular Weight of Hemoglobin.— The relationship between the diffusion coefficient of a substance and the radius of its particle is expressed in the equation:

$$(1) \qquad D = \frac{RT}{N} \cdot \frac{1^*}{6\,\pi\,r\,\eta}$$

The relationship between the particle size and its molecular weight is expressed by the equation:

$$(2) \qquad M = \frac{4}{3}\,\pi\,r^3\,gN$$

By substituting the value of the observed diffusion coefficient of hemoglobin in equation (1), the radius of the particle was found to be 2.71×10^{-7} cm. From equation (2) the value of 67,200 was calculated for the molecular weight of hemoglobin. This value agrees with that found by Adair (4), and with the value found by Svedberg and Nichols (5).

* In equation (1) D = diffusion coefficient of the substance
R = gas constant = 8.3×10^7 erg.·deg.$^{-1}$·mol^{-1}
T = 280° (absolute) for 7°
N = Avogadro's number = 6.06×10^{23} mol^{-1}
r = radius of the particle, with water of hydration, in cm.
η = viscosity of water at 7°
= 0.01422 gm.·cm.$^{-1}$·sec.$^{-1}$
= 0.01422 erg.·sec.·cm.$^{-3}$
In equation (2) M = molecular weight
r = radius of the particle, in cm.
g = specific gravity of the substance
N = Avogadro's number

III. Determination of Molecular Size of the Carbohydrates from Ovomucoid and from Pneumococcus Type III.

1. Specific Gravity of Carbohydrate from Ovomucoid.—The specific gravity of the carbohydrate from ovomucoid (6) was determined gravimetrically by the displacement of xylene. The value was 1.555.

2. Diffusion Coefficient of Carbohydrate from Ovomucoid.—443 mg. of dried carbohydrate were dissolved in water and the sample was diluted with 0.1 M phosphate buffer so that the final volume was 50 cc. and the final concentration of buffer was 0.05 M and the pH of the solution was 6.81. The solution was cooled to 7°, the cell was filled, and the diffusion was carried out in the usual manner.

TABLE III.

Diffusion Coefficient of Carbohydrate from Ovomucoid 1 Per Cent Solution (pH 6.81) at 7°.

Experiment No.	Time.	K for membrane.	Equivalent cc. carbohydrate diffused.	D cm.² per day.
	days		*Q cc.*	
1	0.683	0.165	0.724	0.175
2	1.088		0.992	0.151
3	0.626		0.594	0.157
4	0.330		0.264	0.132
5	0.652		0.601	0.152
Diffusion coefficient 7°....................................				0.153

The concentration of diffused carbohydrate was determined by the colorimetric method of Tillmans and Philippi (7). This particular method was employed because, in the experiments on the pneumococcus polysaccharide, the quantities diffused were so minute that polariscopic readings were out of the question. We found the method of Tillmans and Philippi to be quite accurate if the usual precautions for colorimetric determinations were regarded, and provided we used the same material in our standard. The results of these diffusion experiments are given in Table III.

The diffusion coefficient at 7° was found to be 0.153 cm.² per day.

3. Determination of Specific Gravity of Soluble Specific Substance of Pneumococcus Type III.—The specific gravity of a dried

Experiment 1, Alundum Cell.
Diffusion Coefficient of Soluble Specific Substance Type III, pH 6.81, and at 7°.

Determination No.	Time.	Diffusate diluted to cc.	Amount used for analysis.	Standard.		Color reading of standard.	Color reading of unknown.	S. S. S. III* in sample.	S. S. S. III in total diffusate.	Equivalent cc. carbohydrate diffused.	D cm.² per day.
	days		cc.	cc.	No.	mm.	mm.	mg.	mg.	Q cc.	
1	0.503	25	3	1	4	40	41.5	0.1445	1.205	0.1205	0.0395
2	0.698	25	3	2	2	30	30.5	0.205	1.70	0.170	0.0403
3	0.407	25	3	1	3	30	30.5	0.115	0.96	0.096	0.0390
4	0.709	25	3	2	2	30	31.5	0.198	1.65	0.165	0.0383
5	0.865	25	3	2	3	30	28.7	0.245	2.04	0.204	0.0389
6	0.319	25	3	2	1	25	27.0	0.0926	0.77	0.077	0.0400
7	0.677	25	3	2	2	30	33.6	0.186	1.54	0.154	0.0378
8	0.330	25	3	2	1	25	26.3	0.095	0.79	0.079	0.0395

Average.. 0.0390

Standards used in Experiment 1.

Standard No.	Cc. of concentrated solution diluted to 100 cc.	S. S. S. III per cc.
		mg.
1	0.50	0.050
2	1.04	0.104
3	1.17	0.117
4	1.50	0.150

* S.S.S. III = soluble specific substance Type III.

TABLE V.

Diffusion Coefficient 1 Per Cent Pneumococcus Polysaccharide Type III at 7°.

Experiment No.	Membrane.	pH	Equivalent cc. of carbohydrate diffused.	Average Q cc. per day for each cell.	Average D cm.² per day for each experiment.	Average D cm.² per day for each cell.
			Q cc. per day			
1	Alundum.	6.81	0.237		0.0391	
2		9.5	0.269		0.0444	
				0.253		0.0417
3	Jena.	6.81	0.316		0.0421	
4		6.81	0.290		0.0386	
5		9.5	0.324		0.0431	
				0.310		0.0412
Diffusion coefficient of S. S. S. III* at 7°......................						0.0415

* S.S.S. III = soluble specific substance Type III.

sample of the sodium salt of Type III soluble specific substance was determined by displacement of xylene at 25°, the average value is 1.7658.

4. Preparation of Solution of Soluble Specific Substance.—1 gm. of the Type III soluble substance, previously dried to constant weight, was dissolved in the minimum amount of N NaOH. The solution was made exactly neutral to litmus with HCl and was then diluted to 100 cc. with buffer so that the final concentration of buffer was 0.05 M and the pH of the solution either 6.81 or 9.50 as desired.

The solutions were cooled to 7°, the cells filled, placed in 10 cc. of 0.05 M buffer, and allowed to diffuse for at least 5 hours. The outside solution was then syphoned off and the amount of soluble substance which had diffused was determined by the colorimetric method of Tillmans and Philippi (7).

The complete analytical results of one diffusion experiment are given in Table IV to show how constant the diffusion coefficient remains.

A summary of the several such experiments is shown in Table V.

5. Radius and Molecular Weight of Carbohydrate from Ovomucoid.—From equation (1) the radius of the particle may be calculated, by substituting the value for the observed diffusion coefficient, and the specific gravity of the carbohydrate.

$$r = \frac{1.235 \times 10^{-8}}{0.153}$$

$$r = 8.08 \times 10^{-8} \text{ cm.}$$

Then from equation (2) $M = 2075$.

This value for the molecular weight of the carbohydrate of ovomucoid is in accord with the value obtained by Levene and Rothen (8).

6. Calculation of Radius and Molecular Weight of Soluble Specific Substance Type III.—From equation (1)

$$r = \frac{1.235 \times 10^{-8}}{D}$$

$$= \frac{1.235 \times 10^{-8}}{0.0415}$$

$$= 2.975 \times 10^{-7} \text{ cm.}$$

The molecular weight of the polysaccharide is calculated from formula (2). $M = 118,000$.

It is of great interest that this bacterial carbohydrate, which plays such an important rôle in the immunology of the pneumo-coccus, has a molecular weight as great as that of most of the proteins. It might be mentioned here that this carbohydrate is a relatively strong acid, and if it were highly ionized in solution, the molecular weight, as we have determined it, would be too low. On the other hand, if the molecule is highly hydrated, the value which we report would be too large.

SUMMARY.

The diffusion coefficient of the specific polysaccharide of Type III pneumococcus has been determined. From this constant a value of 118,000 has been calculated as the molecular weight of the Pneumococcus Type III specific carbohydrate.

The authors wish to express their thanks to both Dr. Northrop and Dr. Anson for their advice and suggestions.

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THE PREPARATION OF THE TYPE-SPECIFIC POLY-SACCHARIDES OF PNEUMOCOCCUS.

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In previous publications (1, 2) from this laboratory methods have been described for the preparation of the specific polysaccharides of the three types of pneumococcus. Hitherto the method of isolation of pure carbohydrates from broth cultures of this microorganism has been accomplished by separating the polysaccharide from a mass of accompanying inert protein degradation products through fractional precipitation with alcohol. The success of this method is dependent upon the assumption that the concentration of alcohol, sufficient to precipitate quantitatively the specific gum, is insufficient to precipitate the accompanying inert nitrogenous constituents. In practice this assumption is not entirely correct, for in order to secure a product essentially free from nitrogen, it has hitherto been necessary to subject the carbohydrate to a great number of precipitations with alcohol, a procedure which is not only laborious but costly as well.

For certain work which will be described at a future date, it was necessary to secure relatively large quantities of these specific polysaccharides. It was found that through the initial elimination of the accompanying proteins and their degradation products from broth culture concentrates, excellent yields of the specific polysaccharides could be secured with far less labor and material. This simplified method is described below in the hope that it may be of service to those who do not care to follow the rather elaborate procedure described in earlier publications.

Method.

I. *Type III Pneumococcus Carbohydrate.*

A. *Preparation of Crude Carbohydrate.*—8 day cultures of Type III pneumococcus grown in meat infusion phosphate broth at pH 7.4, containing 0.5 per cent glucose, were concentrated in lots of 23 liters on the water bath to 0.1 of their original volume. The concentrated broth was precipitated with 1.5 volumes of alcohol. After standing overnight at room temperature the precipitate was centrifuged off. The supernatant liquid was discarded. The precipitate was dissolved in 200 to 300 cc. of water and was again centrifuged. The supernatant liquid containing the carbohydrate was placed in a stock bottle in the ice box. The fraction insoluble in water was washed once by mascerating with 100 cc. of water and centrifuging; this supernatant liquid was also placed in the stock bottle. The water-insoluble precipitate, now containing only very little specific carbohydrate, was once again washed and centrifuged, and this supernatant liquid was saved to dissolve the precipitate of carbohydrate from the next alcoholic precipitation.

The clear solution of carbohydrate collected from 175 liters of pneumococcus broth was now at a volume of about 4 liters. The crude carbohydrate was precipitated from this solution with 1.4 to 1.5 volumes of alcohol. A heavy precipitate, containing all of the specific polysaccharide, and a great deal of protein and protein degradation products, separated on standing.

B. *Purification of the Carbohydrate.*—The precipitate was now dissolved in 1000 cc. of water. When all the material had gone into solution, the substance was treated with about 100 cc. of a saturated solution of trichloroacetic acid. A heavy precipitate of coagulated proteins, etc. separated. After standing for 30 minutes this precipitate was centrifuged. The clear supernatant liquid was saved. The precipitate of protein was stirred with 500 cc. of water and was brought into solution by the careful addition of 30 per cent NaOH. The protein was then again precipitated by the addition of 50 cc. of saturated trichloroacetic acid solution. The precipitate containing no specific carbohydrate was now finally centrifuged off and discarded. The supernatant liquid was added to the above. The solution containing the carbohydrate was neutralized with 40 per cent sodium hydroxide. A

small amount of inactive material separated from the solution on standing overnight in the ice box. This was centrifuged off and discarded.

The specific polysaccharide was now precipitated by the addition of 1.5 volumes of alcohol. The carbohydrate flocked out as a large white amorphous mass. After standing overnight this material was separated from the colored mother liquid by centrifugation. The polysaccharide, when dissolved in water gave a solution which was colored a pale yellow. It was again precipitated with alcohol. After this precipitation, the supernatant liquid was nearly colorless. The carbohydrate was dissolved in 400 cc. of water, chilled to 0°, and to the solution were added 100 cc. of 1:1 hydrochloric acid. After standing on ice overnight the material was separated by centrifugation, dissolved again in 400 cc. of water plus sufficient alkali to bring it into solution, and again precipitated by the slow addition of hydrochloric acid. In this manner a snow-white flocculent precipitate was obtained which was finally filtered on a hardened paper, washed free from chloride with redistilled 50 per cent alcohol. It was finally washed with absolute alcohol and ether. 34.5 gm. of polysaccharide were recovered from 175 liters of broth culture. The material contained no ash. It had an optical rotation of $[\alpha]_D^{25°} = -33.2°$. Its acid equivalent was 346. It contained 0.13 per cent of nitrogen. It reacted with antipneumococcus serum in dilutions of 1:6,000,000. It will be noted that the yield of material is exceptionally high as compared with the yield described by previous methods. The properties of the material agree in all respects to those of other preparations hitherto described.

II. Type I Pneumococcus Carbohydrate.

This material was collected and purified exactly as was the carbohydrate from the Type III pneumococcus. Instead of precipitating the carbohydrate with 1:1 hydrochloric acid as was the Type III material, the product, after treatment with trichloroacetic acid and after two precipitations with alcohol, was dissolved in 400 cc. of water. The solution was then chilled to 0° and to it were added 200 cc. of alcohol. After standing in the ice box overnight the carbohydrate was centrifuged off. This precipitation was repeated. The solution of the polysaccharide containing an excess

of HCl was finally dialyzed in a parchment sack against distilled water until free from chlorides, whereupon the specific polysaccharide precipitated out. The carbohydrate was sucked dry on a hardened paper and washed with alcohol and ether. From 350 liters of pneumococcus broth culture 18.5 gm. of specific material were isolated. The analysis of the polysaccharide was identical with those of previous preparations. It had an optical rotation of +300°, a nitrogen content of 5.1 per cent, and amino nitrogen content of 2.5 per cent, it contained no ash, it yielded 29 per cent of reducing sugars on hydrolysis, and it reacted with antipneumococcus serum in dilutions of 1:6,000,000.

SUMMARY.

A method for the preparation of the specific polysaccharides of Type I and Type III pneumococcus has been described.

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SOME OBSERVATIONS ON THE ACTION OF ALKALI UPON CYSTINE AND CYSTEINE.*

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The decomposition of cystine by alkaline reagents has received much study, particularly in recent years by Gortner and his collaborators (1) and by Andrews (2). Consideration of the results obtained by these workers leads to the conclusion that the process is far from simple, apparently consisting of a series of successive reactions, possibly accompanied by simultaneous side reactions. The experiments here reported are, in the main, of an orientating character only, having been carried out in the hope of disclosing fresh lines of attack on the general problem.

Fate of the Organic Portion of the Molecule.—Investigation of the alkaline decomposition products of cystine and of certain of its derivatives led Baumann and others (3) to the conclusion that the initial product is pyruvic acid,[1] which subsequently breaks down under the influence of the alkaline reagent. We have been able to confirm this by the isolation of pyruvic acid *p*-carboxyphenyl-hydrazone (which is stable towards alkali) in yields of over 60 per cent from cystine by heating with alkaline plumbite in the presence of sodium phenylhydrazine-*p*-carboxylate. The same product is obtained in somewhat better yield from cysteine under similar conditions. The phenylhydrazone of pyruvic acid can also be

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[1] It is interesting to note that Dewar and Gamgee (4) reported the formation of pyruvic acid by the action of nitrous acid upon cystine. Since we have been able to show that pyruvic acid gives rise to a small but definite amount of nitrogen in the Van Slyke amino nitrogen procedure, this may furnish a possible explanation of the well known fact that cystine gives abnormally high results in the Van Slyke process.

obtained, though less readily, when the reaction is carried out in the presence of phenylhydrazine in the place of its carboxylic acid. Repeated attempts to isolate hydrazones of pyruvic acid from reaction mixtures containing cystine with calcium hydroxide in the place of sodium hydroxide have consistently failed; cysteine, on the other hand, under these conditions furnishes a high yield of pyruvic acid *p*-carboxyphenylhydrazone.

Further evidence of the production of pyruvic acid by the alkaline decomposition of cystine, both with and without plumbite, is furnished by the development of an intense orange-yellow color when the reaction is carried out in the presence of salicylaldehyde. This test, which is delicate though not specific for pyruvic acid, undoubtedly depends on a reaction analogous to the condensations of salicylaldehyde with acetone (5) and of benzaldehyde with pyruvic acid (6). The product, which is soluble in water, is orange-yellow in alkalies, almost colorless in weak or dilute acids, and lemon-yellow in concentrated hydrochloric acid. A faintly acid solution readily gives up the substance to ether, from which it may again be extracted by dilute alkali and by concentrated hydrochloric acid. The reaction can be followed colorimetrically if the match be made through a light filter which transmits only a narrow band of the spectrum around 495 $m\mu$, at which wavelength the absorption of alkaline solutions of salicylaldehyde is negligible. The method, however, appears unreliable for quantitative purposes.

The succession of reactions whereby pyruvic acid is formed during the decomposition of cystine and cysteine by alkali, outlined by Bergmann and his collaborators (7), is in complete accord with our results. The observation that sulfur and nitrogen appear to be eliminated in approximately equivalent amounts suggests that the second stage (hydrolysis of α-aminoacrylic acid) proceeds with much higher velocity than the first, which therefore determines the rate of the reaction as a whole.

$$
\begin{array}{ccc}
CH_2SNa \cdot & & CH_2 \\
| & & \| \\
CHNH_2 & + NaOH = & CNH_2 & + Na_2S + H_2O \text{ (Slow reaction)} \\
| & & | \\
CO_2Na & & CO_2Na
\end{array}
$$

$$
\begin{array}{ccc}
CH_2 & & CH_3 \\
\| & & | \\
CNH_2 & + H_2O = & CO \quad + NH_3 \text{ (Rapid reaction)} \\
| & & | \\
CO_2Na & & CO_2Na
\end{array}
$$
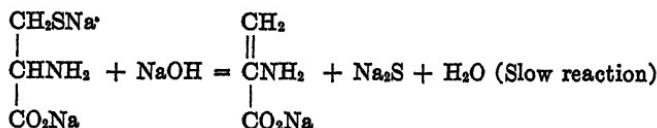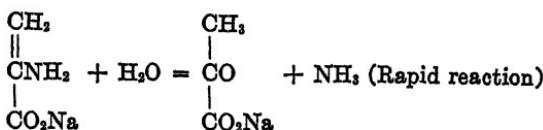
This mechanism finds a partial analogy in the well known conversion by alkali of α,β-dibromopropionic acid into α-bromoacrylic acid.

$$
\begin{array}{ccc}
CH_2Br & & CH_2 \\
| & & \| \\
CHBr & + NaOH = & CBr \quad [+ NaBr + H_2O \\
| & & | \\
CO_2Na & & CO_2Na
\end{array}
$$
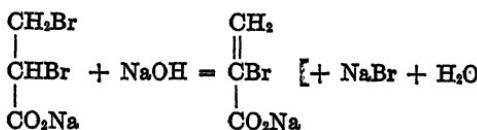
When the dibromo acid is boiled with salicylaldehyde in dilute alkali, the solution acquires the orange-yellow color characteristic of the reaction product of salicylaldehyde with pyruvic acid. This can be explained by the hydrolysis of α-bromoacrylic acid.

$$
\begin{array}{ccc}
CH_2 & & CH_3 \\
\| & & | \\
CBr & + NaOH = & CO \quad + NaBr \\
| & & | \\
CO_2Na & & CO_2Na
\end{array}
$$
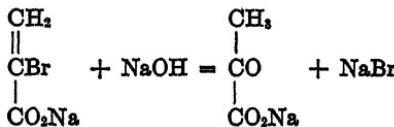
The intensity of the color so formed amounts to less than 7 per cent of that obtainable from cysteine under similar conditions, so that the formation of pyruvic acid constitutes merely a side reaction in the alkaline hydrolysis of the dibromopropionic acid.[2]

Availability of Labile Sulfur in Cystine and Cysteine.—Some doubt arises, after reviewing the literature, as to the proportion of the sulfur in cystine and in cysteine which can be obtained in the form of lead sulfide. Suter (9) stated that 83 per cent could be so

[2] β-Chlorolactic acid, in the form of its ethyl ester (8), gives a pure yellow color, the intensity of which is equivalent to about 12 per cent of the maximum obtainable from cysteine. With glycerol α-chlorohydrin, on the other hand, a dull yellow color of very low intensity is developed only after many hours at 100°. *dl*-Serine (a sample of which was kindly furnished by Dr. H. D. Dakin) develops the yellow color slowly, but no color is obtained with glyceric acid.

No attempt has yet been made to characterize the yellow substance produced in these reactions, beyond noting that it forms a colorless lead salt which is insoluble in dilute acetic acid.

obtained from cystine; Schulz (10) reported a similar figure but stated that only 52 per cent of the sulfur can be obtained from cysteine. Mörner (11) reported that 75 per cent, on the average, of the total sulfur of cystine can be recovered as lead sulfide.

We find that cystine can yield 75 to 80 per cent of its sulfur in the form of lead sulfide, the balance appearing mainly to take the form of sodium thiosulfate;[3] the sulfur of cystine thus seems to be initially eliminated as disulfide. On this interpretation the amount theoretically obtainable as lead sulfide is 75 per cent.

$$4 \, Na_2S_2 + 6 \, Pb(OH)ONa + 3 \, H_2O = 6 \, PbS + Na_2S_2O_3 + 12 \, NaOH$$

Cysteine, on the other hand, furnishes a yield of lead sulfide equivalent to its total sulfur content. The same result is obtained on decomposing cystine with alkaline plumbite in the presence of a suitable reducing agent, such as arsenite or stannite.

The observation that more than 75 per cent of lead sulfide is produced under certain circumstances from cystine can be explained by assuming a partial reduction of the disulfide linkage by the organic moiety of the cystine molecule or by added reducing substances. This effect is particularly noticeable in the presence of salicylaldehyde (see below), when over 90 per cent of the sulfur appears as lead sulfide.

Rate of Elimination of Nitrogen and Sulfur.—A series of measurements was made on the rate of liberation of ammonia at the boiling temperature, employing 0.025 M solutions of cystine in 1 N sodium hydroxide and in suspensions of calcium hydroxide, with and without the addition of lead oxide or acetate. The operation was carried out in a current of hydrogen, thereby excluding air and carrying off the ammonia as fast as it formed. The results of typical experiments are shown in Fig. 1. These confirm the observation of Gortner and Hoffman (1) that deamination is much more rapid with calcium hydroxide than with sodium hydroxide. They also indicate that in both cases the rate is increased (though to different extents) by the addition of lead hydroxide, as has been demonstrated by Andrews (2) for the case of sodium hydroxide.

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[3] We have not attempted to prove the formation of sodium thiosulfate by actual isolation, but have been satisfied to observe the amounts of sulfur, sulfur dioxide, and sulfuric acid formed on acidifying the filtrate from the lead sulfide.

The deamination of cysteine (Fig. 2) by boiling alkaline solutions takes place with considerably lower velocities than those observed with cystine under similar conditions, an effect, recently recorded by Andrews (2), which will be discussed from a theoretical standpoint in a later communication.   The slight augmentation of the initial velocity of ammonia formation may be ascribed to the alkaline decomposition of some of the cystine before the
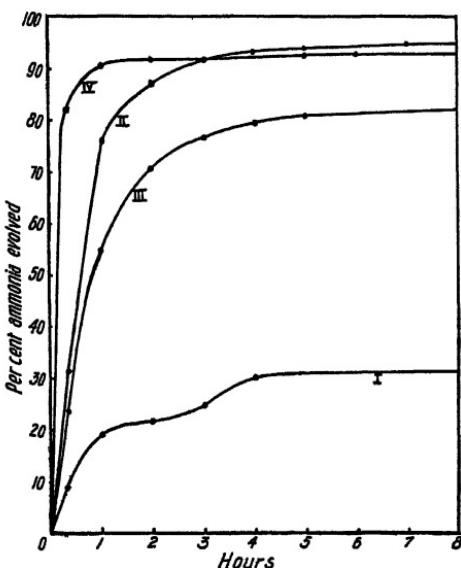
FIG. 1. Curve I, 0.025 M cystine in boiling 1 N sodium hydroxide; Curve II, 0.025 M cystine in a boiling 5 per cent suspension of lime; Curve III, 0.025 M cystine + 0.073 M litharge in boiling 1 N sodium hydroxide; Curve IV, 0.025 M cystine + 0.1 M litharge in a boiling 5 per cent suspension of lime.

reduction by stannite is complete, or of cystine present in the sample of cysteine hydrochloride employed.   Of particular interest is the character of the curve depicting the elimination of ammonia from cysteine with a boiling suspension of lime; the reaction increases in velocity during its progress, furnishing an S-shaped type of curve resembling those of autocatalytic reactions.

Addition of salicylaldehyde to the sodium hydroxide greatly increases the rate of deamination (Fig. 3).   The nature of the

reaction leading to this enhanced reactivity remains to be explained; it appears to be due to the presence of the aldehydic group since, as will be shown below, analogous effects can be induced by the addition of other carbonyl compounds to the reaction mixture. Salicylaldehyde exerts at least two interesting effects on the decom-
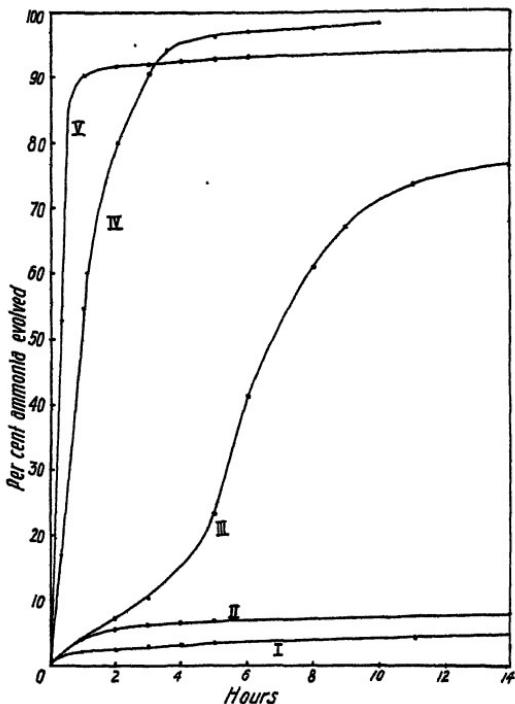
FIG. 2. Curve I, 0.025 M cystine + 0.05 M sodium stannite in boiling 1 N sodium hydroxide; Curve II, 0.05 M cysteine in boiling 1 N sodium hydroxide; Curve III, 0.05 M cysteine in a boiling 5 per cent suspension of lime; Curve IV, 0.05 M cysteine + 0.073 M litharge in boiling 1 N sodium hydroxide; Curve V, 0.05 M cysteine + 0.073 M litharge in a boiling 5 per cent suspension of lime.

position of cystine, apart from increasing the velocity of ammonia formation and combining with the resulting pyruvic acid to form a yellow product. Thus, it permits the liberation of all of the nitrogen as ammonia, apparently inhibiting side reactions wherein the amino group rearranges into more stable forms of combination.

It also reacts with cystine in an acid medium, yielding products from which hydrogen sulfide is readily liberated by warming, even in the absence of lead acetate.

In all of the experiments on the elimination of ammonia at the boiling temperature, except those with mixtures containing salicylaldehyde, the formation of ammonia proceeds very much more slowly towards the end of the reaction. The level at which the curves become nearly horizontal depends roughly upon the initial
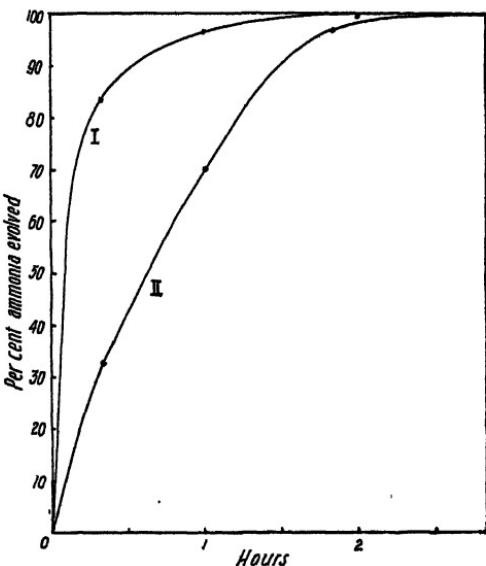
FIG. 3. Curve I, 0.025 M cystine + 0.1 M salicylaldehyde in boiling 1 N sodium hydroxide; Curve II, 0.05 M cysteine + 0.1 M salicylaldehyde in boiling 1 N sodium hydroxide.

velocity, being the highest with the more rapid reactions. This behavior may be regarded as evidence of the formation of secondary decomposition products which break down more slowly than the original cystine at rates apparently uninfluenced by the presence of lime or of lead oxide.

Attempts to correlate the elimination of the sulfur with that of the nitrogen at the boiling temperature were ineffectual owing, on the one hand, to the high speed of the reaction in the presence of plumbite, and on the other, to the loss of hydrogen sulfide from

the boiling alkaline solutions containing no lead.   The indications were, however, that the sulfur is split off at rates at least equal to those at which ammonia is evolved.   The decomposition was accordingly studied at 25°, at which temperature the formation of lead sulfide proceeds with measurable velocity.   As can be seen in Fig. 4, the formation of lead sulfide follows a course not unlike that of an autocatalytic reaction; since the low initial velocity is
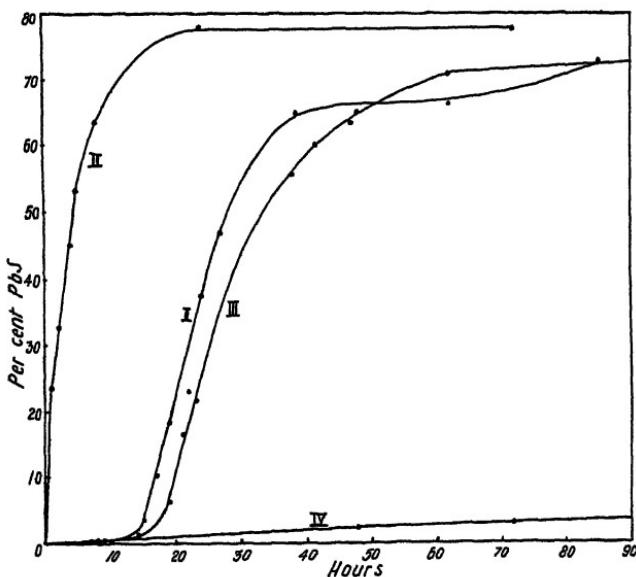
FIG. 4. Curve I, 0.025 M cystine + 0.073 M litharge in 1 N sodium hydroxide at 25°; Curve II, 0.025 M cystine + 0.073 M litharge + 0.144 M sodium pyruvate in 1 N sodium hydroxide at 25°; Curve III, 0.025 M cystine + 0.073 M litharge + 0.7 M ammonia in 1 N sodium hydroxide at 25°; Curve IV, 0.025 M cystine + 0.073 M litharge + 0.13 M sodium p-hydrazinobenzoate in 1 N sodium hydroxide at 25°.

maintained without increase in the presence of sodium p-hydrazinobenzoate and the maximum velocity may be induced by the addition of sodium pyruvate, it appears highly probable that the accelerating agent is the pyruvate formed in the reaction.   The ammonia produced can have little effect on the velocity, since inclusion of 14 equivalents of ammonia in the mixture only slightly depresses the rate of reaction, the "autocatalytic" character of

which remains unimpaired.   An effect similar to that of sodium
pyruvate can also be induced by the addition of benzaldehyde, and
even more markedly by salicylaldehyde (Fig. 5).   Formaldehyde
has a small but appreciable accelerating effect on the reaction in
its initial stages but appears to suppress the subsequent increase
in rate of precipitation of lead sulfide.
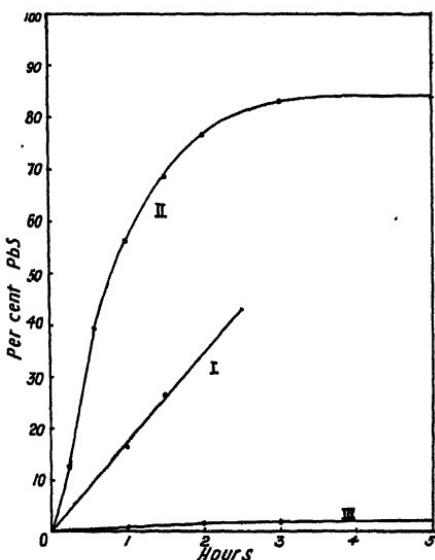
FIG. 5.   Curve I, 0.025 M cystine + 0.073 M litharge + 0.05 M benzaldehyde
in 1 N sodium hydroxide at 25°; Curve II, 0.025 M cystine + 0.073 M litharge
+ 0.05 M salicylaldehyde in 1 N sodium hydroxide at 25°; Curve III, 0.025
M cystine + 0.073 M litharge + 0.25 M formaldehyde in 1 N sodium hydroxide
at 25°.

The deamination experiments carried out at 25° differ from
those hitherto considered in that the ammonia originally produced
remained in the reaction mixture until decomposition was checked
by the addition of acid.   During the early stages of the reaction
the amount of ammonia liberated corresponds closely to the
amount of lead sulfide precipitated; later, the quantity present
becomes stationary at a level below that reached by the sulfide,
and in most cases subsequently falls (Fig. 6).   This points to the
occurrence of a recombination of ammonia with some decomposi-

tion product of the cystine.   The resultant product is apparently decomposed by alkali at a very slow rate, which is not appreciable at 25° and just measurable at 100°.   Pyruvic acid appears to play only a minor rôle in this reabsorption of ammonia, since the addition of sodium pyruvate has little effect on the ratio of nitrogen to sulfur mobilized, the reaction proceeding from the outset at its maximum velocity with respect to both elements.
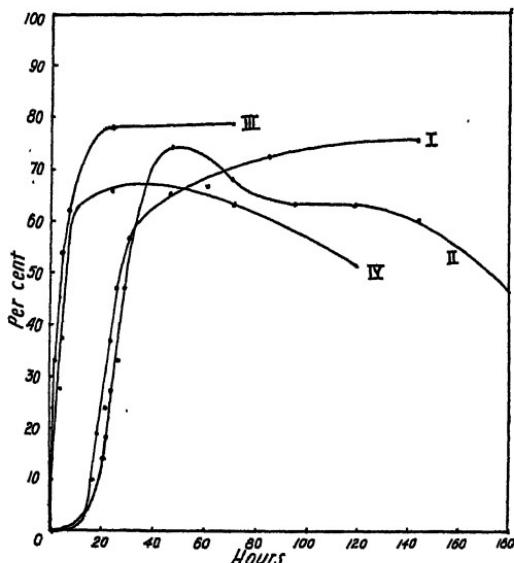
Fig. 6. Curve I, PbS from 0.025 m cystine + 0.073 m litharge in 1 n sodium hydroxide; Curve II, NH₃ from 0.025 m cystine + 0.073 m litharge in 1 n sodium hydroxide; Curve III, PbS from 0.025 m cystine + 0.073 m litharge + 0.144 m sodium pyruvate in 1 n sodium hydroxide; Curve IV, NH₃ from 0.025 m cystine + 0.073 m litharge + 0.144 m sodium pyruvate in 1 n sodium hydroxide.

In the course of experiments on the action of alkali alone at 25° the unexpected observation was made that during the first stage of the decomposition none of the sulfur appears as sulfide ion.   If at any time during the first 2 to 3 days lead acetate or sodium plumbite solution be added, a perfectly clear solution results which, however, soon becomes turbid.   If this clear solution be acidified with acetic acid, an amorphous, reddish brown precipitate sepa-

rates. This can be collected by centrifuging; on treatment with alkali it rapidly turns black owing to the formation of lead sulfide, the alkaline solution being colored light yellow by a soluble organic compound. A similar change occurs more slowly in the absence of alkali. It has so far been found impracticable to study this compound, owing to its instability, and no attempt will here be made to offer an explanation. It is of interest, however, to note that the rate at which the sulfur is thus mobilized in cystine by 1 N sodium hydroxide alone is nearly equivalent to that at which ammonia is liberated in the same solution.

As in the other cases studied, reduction of the disulfide linkage by alkaline stannite causes a pronounced decrease in velocity of decomposition.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Pyruvic Acid p-Carboxyphenylhydrazone.*</div>

From Cystine.—A mixture of 8 gm. of lead acetate in 300 cc. of water, 200 cc. of 1 N sodium hydroxide, 3.1 gm. (0.02 mol) of *p*-hydrazinobenzoic acid, and 2.4 gm. (0.01 mol) of cystine was boiled under reflux for 16 hours. At the end of that time the lead sulfide was removed and the filtrate acidified with 6 cc. of glacial acetic acid, again filtered, treated with 10 cc. of 28 per cent ammonia, and filtered a third time. The clear solution so obtained was evaporated to dryness on the water bath, redissolved in boiling ammonia, filtered, and strongly acidified with hydrochloric acid. The yellow precipitate was collected, well washed, and dried; weight, 2.8 gm. or 63 per cent of the theoretical amount. On recrystallization from 50 cc. of hot 95 per cent ethyl alcohol it separated as light yellow leaflets which melted at 257° (corrected) with evolution of gas but without darkening. On dissolving in dilute ammonia and acidifying the hot solution with acetic acid, it separated slowly in pale orange leaflets which melted with decomposition at 259° (corrected).

From Cysteine.—To a boiling mixture of 8 gm. of lead acetate, 220 cc. of 1 N sodium hydroxide, 275 cc. of water, and 3.1 gm. (0.02 mol) of *p*-hydrazinobenzoic acid were added 3.3 gm. (0.02 mol based on the analysis) of cysteine hydrochloride. After boiling under reflux for 18 hours the crude product was isolated in the manner above described; the yield was 3.8 gm. or 86 per cent of

the theoretical amount. Recrystallization was effected by acidifying with acetic acid a hot solution in dilute ammonia; yield, 3.1 gm. The properties were identical with those of the substance described above.

Analysis.—4.679 mg. gave 0.153 cc. of nitrogen at 27° and 765 mm., whence N = 12.56 per cent. Neutral equivalent = 110.2. $C_{10}H_{10}O_4N_2$ requires N = 12.61 per cent; neutral equivalent = 111.

In another experiment a solution of 3.1 gm. of *p*-hydrazinobenzoic acid in 20 cc. of water containing just enough sodium hydroxide for complete solution was added to a boiling suspension of 10 gm. of calcium oxide and 5 gm. of purified litharge in 180 cc. of water. A solution of 3.3 gm. of cysteine hydrochloride in 50 cc. of water was then added. The mixture immediately became pasty, apparently owing to the separation of an insoluble complex. After boiling under reflux for 24 hours this original precipitate had become entirely replaced by lead sulfide, which was filtered off and washed with dilute potassium carbonate solution. The filtrates were concentrated and treated with a slight excess of potassium carbonate; the calcium carbonate was removed and the clear filtrate concentrated to 100 cc. This was allowed to cool and was then acidified with hydrochloric acid. The yellow precipitate was collected and washed successively with dilute hydrochloric acid, water, and a little cold alcohol. The yield was 4.5 gm. of a very pale yellow solid which melted at 251° (corrected) with decomposition. On treating with dilute ammonia, 0.7 gm. of lead oxide remained undissolved. The filtrate was acidified with acetic acid, when typical pale yellow leaflets slowly separated in a yield of 3.0 gm. The product melted at 253° (corrected) and showed a neutral equivalent of 109.5.

From Pyruvic Acid.—The same compound was obtained on adding 1.5 cc. of pyruvic acid to a solution of 3.1 gm. of *p*-hydrazinobenzoic acid in 50 cc. of water and 85 cc. of 1 N sodium hydroxide and warming at 90–95° for 90 minutes. It melted at 258.5° (corrected).

Pyruvic Acid Phenylhydrazone.

A mixture of 6 gm. of lead acetate, 85 cc. of 1.0 N sodium hydroxide, 5.4 gm. of phenylhydrazine, 2.4 gm. of cystine, and a few drops

of butyl alcohol (to prevent foaming) was boiled for 48 hours under reflux. The odor of benzene developed during the reaction. The lead sulfide was removed and the filtrate acidified with acetic acid. The yellow crystalline precipitate was collected and recrystallized from alcohol, from which the product separated in long, yellow needles which melted with decomposition at 191° (corrected). The yield was 1.4 gm. A further small quantity of the same crystals was secured from the mother liquor, together with a yellow amorphous by-product and traces of an oil possessing an odor like that of acetophenone.

Behavior of Pyruvic Acid towards Nitrous Acid.

Solutions of different amounts of pyruvic acid in 2 cc. of water were treated in the Van Slyke amino nitrogen apparatus according to the standard procedure.

Pyruvic acid.	Time of reaction.	Apparent amino nitrogen.
mg.		*mg.*
10	5 min.	0.208
10	2 hrs.	0.205
40	1 hr.	0.490
40	2.5 hrs.	1.050
40	4.5 "	1.005

Color Test for Pyruvic Acid.

A solution of 1.06 cc. (0.01 mol) of salicylaldehyde and 1 cc. of pyruvic acid (Eastman Kodak Company) in 50 cc. of 2 N sodium hydroxide was warmed on the steam bath for 2 hours. At the end of this time examination of a small sample showed the presence of unchanged salicylaldehyde. A further 1.6 cc. of pyruvic acid was therefore added and heating continued for a half hour longer, when the reaction appeared to be complete. 1 cc. of the resulting solution was diluted to 50 cc. and its color intensity was found to be 85 per cent of that of a 0.01 M potassium dichromate solution, using a Wratten light filter No. 75, whence, on a molar basis, the color intensity is equivalent to that of 2.12 M dichromate. Repetition of this experiment with twice the quantity of pyruvic acid gave a color of considerably lower intensity, indicating the probability that side reactions occur and render the test unsuitable for accurate quantitative measurements.

From Cystine.—A solution of 0.600 gm. (0.0025 mol) of cystine and 1.06 cc. (0.01 mol) of salicylaldehyde in 100 cc. of 1 N sodium hydroxide was allowed to stand at 25°. At the end of 20 minutes the color had already changed appreciably, and a 10 mm. layer matched 20.8 mm. of 0.01 M potassium dichromate. At the end of 19 hours 1 cc. of this solution was diluted to 25 cc.; a 20 mm. layer matched 21.4 mm. of 0.01 M dichromate, whence the molar intensity was equivalent to 5.35 M dichromate.

A similar mixture after boiling for 4 hours showed a color intensity, on a molar basis, corresponding to 6.175 M dichromate.

From Cysteine.—A similar solution containing a corresponding quantity of cysteine hydrochloride, after being boiled for 5 hours, showed a molar color intensity equal to that of 6.7 M dichromate; in a duplicate experiment, however, a color intensity of only 4.74 was obtained.

From α,β-Dibromopropionic Acid.—A mixture of 2.3 gm. (0.01 mol) of α,β-dibromopropionic acid and 1.06 cc. (0.01 mol) of salicylaldehyde in 30 cc. of 2 N sodium hydroxide and 20 cc. of 1 N sodium hydroxide was boiled for 5 hours. The color intensity was equivalent to that of 0.092 M dichromate, corresponding, on the molecular basis, to 0.46 M dichromate.

From β-Chlorolactic Acid.—A mixture of 1.525 gm. (0.01 mol) of crystalline ethyl β-chlorolactate (8) and 1.06 cc. (0.01 mol) of salicylaldehyde in 20 cc. of 2 N sodium hydroxide and 30 cc. of 1 N sodium hydroxide was boiled under a reflux. Samples were withdrawn at intervals, suitably diluted, and matched against dichromate.

After 1 hour the color was equal to that of 0.062 M dichromate; after 2 hours, 0.0825 M; after 4.5 hours, 0.079 M. The color intensity thus passes through a maximum corresponding, on a molar basis, to 0.412 M dichromate.

Determination of Labile Sulfur.

The decomposition of the cystine and cysteine was effected by boiling under reflux a standard sodium hydroxide solution or a suspension of lime, to which had been added more than a sufficient quantity of lead acetate solution, in a current of hydrogen until free of air, then adding the accurately weighed sample, washing it in with freshly boiled distilled water. In order to avoid contam-

ination by rubber stoppers, ground glass joints were exclusively employed in the apparatus. Boiling was continued until the formation of lead sulfide had ceased for some hours; the mixture was allowed to cool in an atmosphere of hydrogen.

The lead sulfide was collected, thoroughly washed, and replaced in the reaction vessel, together with freshly boiled water. This suspension was then boiled in a current of hydrogen until all air had been displaced; 20 per cent hydrochloric acid was then added slowly through the dropping funnel, and the hydrogen sulfide thus liberated was absorbed in a solution of pure sodium hydroxide. The alkaline sodium sulfide was oxidized by means of bromine, followed by potassium chlorate and hydrochloric acid, and the resulting sulfate estimated as barium sulfate. In almost all cases it was found impossible entirely to avoid oxidation of lead sulfide during collection and washing, with the result that small amounts of sulfur remained in the decomposing flask. The acid residue was therefore boiled with about one-third of its volume of ethylene chloride; this readily dissolved the sulfur. The oily layer was oxidized with bromine, chlorate, and hydrochloric acid, and the resulting sulfate estimated as barium sulfate.

The filtrate from the lead sulfide was now placed in the apparatus, concentrated to a small volume in a current of hydrogen, and acidified with hydrochloric acid after restoring the cold water to the reflux condenser. Sulfur dioxide was evolved and collected in sodium hydroxide solution. It was oxidized by bromine and estimated as barium sulfate. Sulfur separated from the residual liquid and deposited to some extent in the lower part of the condenser. It was likewise dissolved in boiling ethylene chloride and oxidized. The residual acid solutions contained small quantities of sulfate, which also was estimated as the barium salt.

In certain cases the sulfur was collected together with the sulfur dioxide, by taking advantage of its volatility with steam.

Cystine with Lime.—To a boiling mixture of 5 gm. of calcium oxide, 100 cc. of water and 35 cc. of a 10 per cent solution of crystallized lead acetate, there was added 1.0000 gm. of cystine in 17 cc. of 0.75 N sodium hydroxide, followed by three 10 cc. portions of water. After 6 hours boiling, the ammonia evolved amounted to 88.9 per cent of the theoretical amount, and after a total of 14 hours this figure had increased to 89.06 per cent. A further 0.55

per cent was obtained on concentrating the filtrate from the lead sulfide, making 89.2 per cent in all.

The distribution of the sulfur was as follows: from lead sulfide, 84.5 per cent; from sulfur dioxide and sulfur, 4.6 per cent; as sulfate in the residue, 1.4 per cent.

In a similar experiment, 94.3 per cent of the theoretical amount of ammonia was evolved during 29 hours boiling, and 5.2 per cent more was recovered (by Kjeldahl digestion) from the filtrate after estimating the various sulfur fractions. These were distributed as follows: hydrogen sulfide from lead sulfide, 82.9 per cent; sulfur with lead sulfide, 1.9 per cent; sulfur dioxide from filtrate, 1.1 per cent; sulfur from filtrate, 0.3 per cent; sulfuric acid from filtrate, 0.3 per cent.

Cystine with Lime with p-Hydrazinobenzoic Acid.—This was carried out similarly to the above, except that 18.1 cc. of 0.5 M sodium p-hydrazinobenzoate were included. After 37 hours boiling, 100.0 per cent of the calculated amount of ammonia had been evolved. The distribution of the sulfur was as follows: hydrogen sulfide from lead sulfide, 81.9 per cent; sulfur with lead sulfide, 6.0 per cent; sulfur dioxide from filtrate, 2.4 per cent; sulfur from filtrate, 0.9 per cent; sulfuric acid from filtrate, 0.5 per cent.

Cystine with Sodium Hydroxide and Stannite.—To a boiling solution of 2.0 gm. of crystallized stannous chloride in 100 cc. of 1 N sodium hydroxide was added a solution of 1.0002 gm. of cystine in 15 cc. of 1 N sodium hydroxide, followed by 15 cc. of 1 N sodium hydroxide. After 45 minutes boiling, a suspension of 4.0 gm. of pure litharge in 25 cc. of 1 N sodium hydroxide was added, and the mixture was heated for 96 hours in boiling water.[4] The lead sulfide gave no free sulfur on decomposition with hydrochloric acid; 99.1 per cent of the calculated amount of hydrogen sulfide was obtained. The acid solution contained metallic lead, formed by the reduction of plumbite by the unoxidized excess of stannite. In a parallel experiment, heated for 120 hours, 98.5 per cent of the calculated amount of hydrogen sulfide was obtained; a minute amount of sulfur was observed to have collected in the condenser.

[4] This method of heating was necessitated by the violent bumping observed on boiling mixtures of cystine with sodium plumbite solution. Suspensions of lime do not show this tendency.

Cystine with Lime, Sodium Hydroxide, and Arsenite.—A solution of 3.0 gm. of arsenious acid and 0.3330 gm. of cystine in 100 cc. of 0.5 N sodium hydroxide was boiled for 45 minutes, whereupon a suspension of 2 gm. of lime and 2 gm. of litharge in 135 cc. of 0.5 N sodium hydroxide was added and washed in with 15 cc. of water. The mixture was boiled in a current of hydrogen for 24 hours, during which time 96.6 per cent of the calculated quantity of ammonia was evolved. The mixed precipitate was collected and thoroughly washed with dilute alkali in order to remove arsenic (which interferes with the liberation of hydrogen sulfide) as completely as possible. On decomposition with hydrochloric acid, 93.4 per cent of the calculated quantity of hydrogen sulfide was liberated and 1.2 per cent of sulfur was recovered from the condenser, giving a total of 94.6 per cent.

Cysteine with Lime.—The cysteine hydrochloride employed contained 19.35 per cent (95.4 per cent of the theoretical amount) of sulfur and 25.5 per cent of chlorine. On titration with iodine, 81 per cent of the sulfur present was found to be in the sulfhydryl form. A sample of this material weighing 0.9324 gm. was decomposed by boiling for 3 hours with a suspension of 5 gm. of lime in 120 cc. of water containing 2 gm. of lead acetate. The ammonia evolved amounted to 74 per cent of the calculated quantity; 93.2 per cent of the sulfur was recovered as hydrogen sulfide and 0.23 per cent as sulfur dioxide.

Decomposition of Thiosulfate.—Known quantities (25 cc. of 0.06067 N) of sodium thiosulfate solution were treated in the manner described above for the filtrates. The distribution of the sulfur, in a typical experiment, was found to be: as sulfur dioxide, 46.4 per cent; as sulfur, 48.1 per cent; as sulfuric acid, 1.3 per cent.

Rate of Evolution of Ammonia at the Boiling Temperature.

The apparatus consisted of a 2-necked flask to which were attached a dropping funnel, an inlet tube for hydrogen, and an efficient reflux condenser, from the top of which a tube led to an absorption vessel containing standard acid. All connections were of glass. In carrying out a run the flask was charged with all of the reagents except the cystine or cysteine. The mixture was boiled and all of the air carefully swept out with hydrogen, whereupon the cystine was added to the boiling mixture through the

dropping funnel, a definite quantity of boiling water being employed for washing it down. The current of hydrogen was regulated at 2 to 3 bubbles per second and the absorbing acid was removed and replaced at definite intervals.

The total volume of liquid was so adjusted that the cystine was always present in a concentration of 0.025 M and the cysteine in a corresponding concentration, 0.05 M. The concentration of the

TABLE I.

Total Percentages of Theoretically Obtainable Ammonia.

0.025 M cystine + 0.1 M lead acetate in a boiling 5 per cent suspension of lime.

Hrs....................	0.33	1	3	5	7	9	13	29	36	43
Per cent NH$_3$..........	84.5	89.8	90.9	91.6	92.0	92.3	93.5	93.8	94.0	94.2

0.025 M cystine + 0.1 M sodium p-hydrazinobenzoate + 0.073 M litharge in boiling 1 N sodium hydroxide.

Hrs....................	0.33	1	1.5	2
Per cent NH$_3$..........	18.0	43.8	55.8	69.2

0.025 M cystine + 0.05 M sodium p-hydrazinobenzoate in a boiling 5 per cent suspension of lime.

Hrs....................	0.33	1	2	3	5	7	9	11	17	34
Per cent NH$_3$..........	65.5	85.2	85.4	86.4	87.5	87.8	88.0	88.2	88.8	89.6

0.025 M cystine + 0.05 M sodium p-hydrazinobenzoate + 0.1 M lead acetate in a boiling 5 per cent suspension of lime.

Hrs....................	0.33	1	2	3	4	5	6	12	37
Per cent NH$_3$..........	80.9	92.8	93.8	94.3	94.7	95.0	95.3	95.7	100.0

sodium hydroxide was always 1 N, suitable adjustment being made when necessary for the acidity of any added substances and for the hydrochloric acid present in the cysteine hydrochloride. In the experiments with calcium hydroxide an ample excess of lime in suspension was employed.

The experimental results are expressed as total percentages of the theoretically obtainable amounts of ammonia. It may be added that in all cases in which the reaction mixture contained no lead

·xide, hydrogen sulfide began to escape with the ammonia after he reaction had progressed to the extent of about 30 per cent.

Table I relates to experiments the results of which have not been ecorded graphically above.

Rate of Formation of Ammonia and Lead Sulfide at 25°.

As in the experiments at the boiling temperature, 0.025 M olutions of cystine in 1 N sodium hydroxide were employed, 10 cc. ortions being placed in stoppered centrifuge tubes and allowed o stand in a thermostat at 25°. These tubes were removed at

TABLE II.

Experiments at 25°.

0.025 M cystine in 1 N sodium hydroxide.

Days..........................	2	5	9	12	19	23	26	30
Per cent S..................	1.2	20.1	30.0	30.9	31.9	34.1	32.2	35.4
" " NH₃..............	5.5	24.8	36.4	42.7	42.7	38.0	37.5	39.4

0.025 M cystine + 0.05 M stannous chloride in 1 N sodium hydroxide.

Days......................	2	5	7	20	36	73	
Per cent NH₃..............	2.6	4.7	5.3	7.2	9.8	19.0	

.025 M cystine + 0.1 M lead acetate in a 5 per cent suspension of lime.

Hrs.........................	16	17	18	19	21	23	25	40
Per cent S as PbS..........	25.7	31.3	36.0	38.0	43.5	51.1	56.8	71.3
" " NH₃..............	28.1	28.6			34.3		46.7	45.1

ntervals and the lead sulfide collected by centrifuging and wash-ng. When it was desired to estimate the free ammonia present, ;he supernatant liquor was neutralized by the addition of 1 cc. of glacial acetic acid, allowed to stand for 24 hours in the refrigerator, and freed of any cystine that may have crystallized out. An aliquot portion of the clear solution was treated with an excess of solid potassium carbonate and the ammonia determined by aeration.

The sulfur in the lead sulfide was estimated in the following manner. The precipitate in the centrifuge tube, after being thoroughly washed with dilute alkali, was covered with an approxi-

mately equal volume of potassium chlorate crystals, then 6 to 8 cc. of a 10 per cent solution of bromine in 20 per cent hydrochloric acid were added. On cautiously agitating the precipitate with a glass rod the lead sulfide decomposed with liberation of sulfur; the latter could be readily oxidized by the addition of 2 cc. of concentrated hydrochloric acid and gentle warming with agitation. The clear yellow solution was then washed into a beaker with distilled water and evaporated completely to dryness on the water bath. The residue, consisting of lead sulfate and potassium chloride, was dissolved in boiling 0.25 per cent hydrochloric acid and treated with barium chloride in the usual manner.

Table II supplies the records of experiments which do not appear in graphical form.

SUMMARY.

1. The formation of pyruvic acid as an intermediate product in the decomposition of cystine by alkaline plumbite has been confirmed by the isolation of its p-carboxyphenylhydrazone when the reaction is carried out in the presence of a salt of p-hydrazinobenzoic acid. Cysteine yields the same product under similar conditions.

2. Pyruvic acid condenses in alkaline solution with salicylaldehyde, producing a characteristic orange color. This same color is gradually developed on bringing together cystine (or cysteine) and salicylaldehyde in alkaline solution, either alone or in the presence of plumbite. Certain other compounds of analogous structure, notably α,β-dibromopropionic acid and β-chlorolactic acid, respond qualitatively in the same way.

3. Cysteine is capable of yielding all of its sulfur in the form of lead sulfide on being subjected to the action of alkaline plumbite. Cystine, on the other hand, normally yields only 75 per cent, the remainder appearing largely as thiosulfate. Higher values are sometimes obtained, apparently through the reducing influence of decomposition products or of added substances.

4. In the alkaline decomposition of cystine, ammonia is formed in amounts not more, and frequently less, than the corresponding quantities of sulfide. Recombination of ammonia with decomposition products appears to occur, with the formation of substances which are relatively slowly broken up by alkali.

5. The decomposition of cystine is accelerated by the addition of benzaldehyde, salicylaldehyde, and pyruvic acid. This effect is reflected in the S-shaped velocity curve representing the formation of lead sulfide from cystine and alkaline plumbite. Addition of sodium p-hydrazinobenzoate prevents the increase in velocity, presumably by combining with the pyruvic acid as it forms.

6. Indications have been obtained that when cystine reacts with sodium hydroxide solution alone, an unstable product is primarily formed before sulfide ion makes its appearance.

It is a pleasure to acknowledge the valuable assistance rendered in the experimental work by Dr. Letha Davies Behr.

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A STUDY OF THE ANTIMONY TRICHLORIDE COLOR REACTION FOR VITAMIN A.

III. THE EFFECT OF CONCENTRATION OF REAGENT USED, AND THE STABILITY OF THE CHROMOGENIC SUBSTANCE TO LIGHT.

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Effect of Concentration of Reagent.

In previous papers (1) it has been pointed out that if there is a correlation between the colorimetric and biological assays of vitamin A, a comparison can be made only between the blue color produced by the action of a chloroform solution of antimony trichloride on a fish liver oil and the biological assay of vitamin A on the same oil, where the color produced by antimony trichloride is a linear function of the concentration; also that the dilution curve of cod liver oil with antimony trichloride approaches a linear function at low values of blue, so that the tangent to the dilution curve at the origin may be considered as the curve that would be produced if the color were a linear function of the concentration.

Wokes and Willimott (2) have shown that the depth of blue produced by a given cod liver oil is dependent upon the concentration of antimony trichloride reagent used in such a way that when the concentration of antimony trichloride is halved the tintometer readings under the conditions of their method are reduced to about two-thirds the original value and that low concentrations of antimony trichloride make the color change more rapid. They suggest the concentration be kept between 22 to 24 per cent. Drummond and Morton (3) and Ahmad and Drummond (4) used 30 per cent antimony trichloride solution, as originally suggested

by Carr and Price (5), and limit their readings between 5 and 8 blue units. Jones *et al.* (6), using a saturated solution of antimony trichloride with a 30 per cent chloroform solution of cod liver oil and making readings between 5 and 12 blue units, were unable to show a close correlation with feeding experiments.

In order that the test be standardized and comparable results obtained, the authors (1) have adopted the use of antimony trichloride in chloroform saturated at the temperature of ice and water. This gives a solution which is easily reproducible and has the added advantage of being a cool solution, thus decreasing the rate of fading of the blue color. The initial temperature of the solution being always the same, gives comparable results.

A cholesterol-free chloroform solution of the unsaponifiable portion of cod liver oil is a linear function of concentration but ceases to be a linear function at high concentrations, the intensity of the color produced at high concentrations being more dependent upon the concentration of antimony trichloride reagent used than that produced at low concentrations.

To study the influence of the concentration of antimony trichloride reagent upon the color produced, various amounts of cod liver oil and of a solution of the unsaponifiable fraction were treated with reagents of different concentrations. The antimony trichloride used for the reagents was washed with chloroform and dissolved in anhydrous chloroform in varying amounts. The antimony trichloride in each of five solutions was determined by analysis (7) to be 1.7, 4.3, 10.9, 19.2, and 36.3 gm. per 100 cc. of solution. The first four solutions were kept in an ice and water bath and used at that temperature, the 36.3 per cent solution was used at room temperature (24–25°).

A sample of cod liver oil was diluted with anhydrous chloroform to the following concentrations, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 per cent, and colorimetric determinations were made with each solution, the various antimony trichloride reagents being used. The technique outlined in previous papers was used, readings being taken at 30 seconds with a Lovibond tintometer.

The higher temperature of the 36.3 per cent antimony trichloride solution increased the rate of fading with a greater development of red in high concentrations of cod liver oil. From Fig. 1 it can be seen how impossible it would be to make comparisons with solu-

tions of 30 to 35 per cent antimony trichloride giving colors of 12 to 15 units with the sample of cod liver oil studied. Even with color values of 5 to 8 units as suggested by Drummond *et al.* the
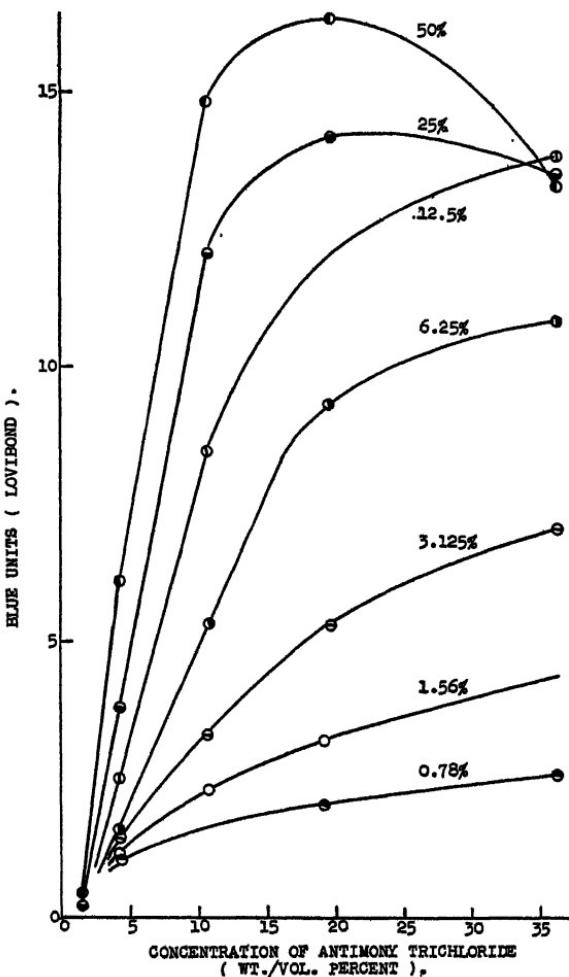
FIG. 1. Color produced by solutions of cod liver oil with various concentrations of antimony trichloride reagent.

variation of color produced with slight variations in concentration of reagent is large. A similar relationship is shown by the values in Fig. 2 where a chloroform solution of the unsaponifiable portion

of cod liver oil from which the cholesterol had not been removed was used. The solution did not show the development of a dark
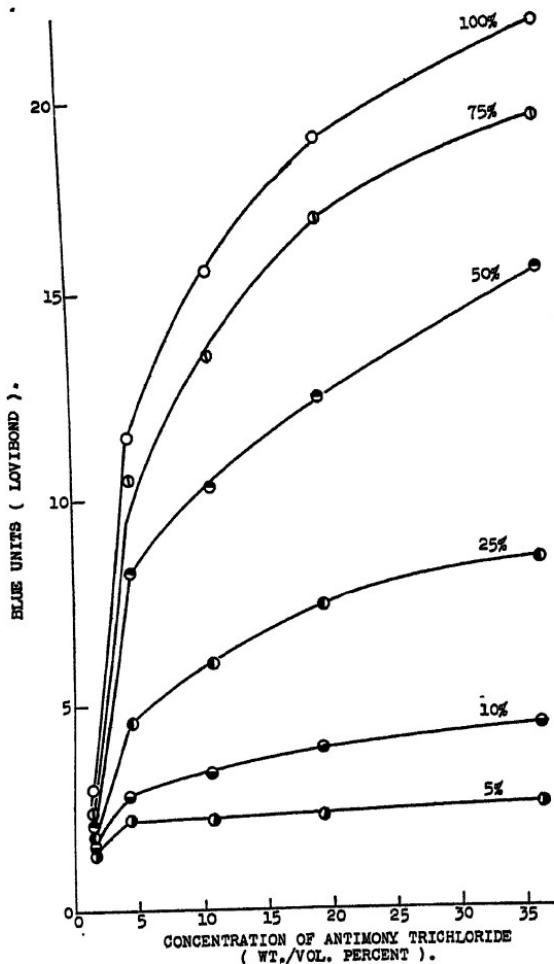
FIG. 2. Color produced by chloroform solutions of the non-saponifiable fraction of cod liver oil with various concentrations of antimony trichloride reagent. Each curve represents the indicated concentration of the non-saponifiable fraction.

red color at high concentrations nor the very rapid fading found in the cod liver oil, but there is still the relatively large variation

in color produced with concentration of antimony trichloride when the readings are above 5 blue units. If the comparison is made at very low color values or by determining the tangent to the dilution curve at the origin the significant values are those below 3 blue units. In this portion of the figure there is very slight difference of observed color with variations of antimony trichloride concentrations. When saturated at the temperature of ice and water the reagent is about 19 per cent.
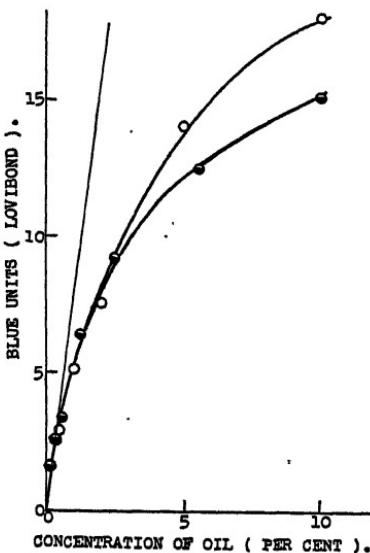
FIG. 3. The influence of the intensity of illumination upon the observed color at 30 seconds with a single sample of cod liver oil. ○——○, reflected light of low intensity. ●——●, direct illumination of higher intensity.

With high concentrations the rate of fading of the color produced and consequently the observed color at 30 seconds is influenced by the intensity of light used in making the observations, as shown in Fig. 3, which gives two dilution curves on the same oil; for one diffused light reflected from a dull white surface was used, for the other a more intense direct light. With dilute solutions where the rate of fading is slow the observed values of the two curves check very closely so that the same values would be obtained from an estimated tangent at the origin.

The observed colors at 30 seconds of the chromogenic substances of three cod liver oils treated with antimony trichloride solution, when illuminated by a direct light and when the same source of light is used but the intensity decreased somewhat by interposing a blue daylight filter, are given in Table I.   The most marked difference is the more rapid development of red with the more intense light, with a tendency to more rapid fading of the blue.

Stability of the Chromogenic Substance to Light.

Drummond, Zilva, and Coward (8) in 1924 stated that cod liver oil should be stored with minimum exposure to air, at as low a temperature as possible, and in the dark.   2 years later Holmes and Pigott (9) recommended light-proof containers in preserving

TABLE I.

Effect of Intensity of Light upon the Observed Color at 30 Seconds.

Oil.	Color observed without filter.			Color observed with daylight filter.		
	B	Y	R	B	Y	R
A (6 per cent).	9.3	1.0	2.5	9.5	1.0	1.5
B (10 ").	15.9	0.9	8.0	18.0	0.9	4.0
C	15.5	1.5	10.0	15.5	1.0	5.5

B, Y, and R refer to Lovibond units of blue, yellow, and red respectively.

vitamin A of cod liver oil.   Peacock (10) the same year showed that cod liver oil after exposure to the carbon arc, mercury vapor lamp, or sunlight failed to give the arsenic trichloride test for vitamin A.   In 1927 Willimott and Wokes (11) studied the action of ultra-violet light upon vitamin A in cod liver oil.   They followed the destruction of the chromogenic substance of cod liver oil when exposed uncovered at a distance of 3 inches from a quartz mercury vapor lamp by means of the color reaction.   They also found that the destruction continued after removal of the samples from exposure to the light, and suggested that the destruction may be due to ozone from the mercury vapor lamp.   Drummond and Morton (3) in 1929 stored samples of cod liver oil in the light and in the dark, with an air space of about 20 cc., for 1 year.   They found loss was generally twice as great in the light as in the dark.

Experiments were performed to determine which wave-lengths of the visible spectrum were most destructive to the chromogenic substance of cod liver oil. A series of four tightly stoppered test-tubes containing a chloroform solution of the non-saponifiable portion of cod liver oil, giving 13.7 blue and 5.9 yellow Lovibond units, was placed behind windows in small cardboard boxes. Fastened over the windows of the boxes were $\frac{1}{2}$ inch cells filled with water, and light filters, such that the first tube, which had no filter, transmitted white light, the second, with a blue filter, transmitted light of wave-length less than 520 $m\mu$; the third, with
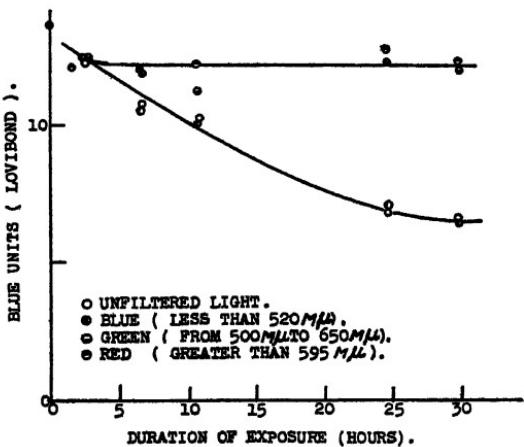
FIG. 4. The effect of light of different wave-lengths on the chromogenic substance of the unsaponifiable portion of cod liver oil.

a green filter, transmitted light of wave-length between 500 and 650 $m\mu$; the fourth, with a red filter, transmitted light of wave-length greater than 595 $m\mu$. The boxes were then placed so that the tubes were 10 inches from a 200 watt tungsten lamp and the room maintained at approximately 24°. Samples were taken from the tubes at intervals and tested colorimetrically. The change in the blue produced is given in Fig. 4. There is marked destruction of the chromogenic substance when exposed to white or blue light and practically no change or only very slight change with the green and red light, showing that the destruction is due to light of wave-length less than 500 $m\mu$. After 30 hours exposure

the tubes were placed in the ice box for 33 days and again tested with the following results in blue units; white 2.3, blue 2.9, green

Effect of Visible Light upon the Stability of Vitamin A in a Chloroform Solution of a Cod Liver Oil (25 Per Cent).

Duration of exposure.		Filter used.												
		None.			Blue.			Green.			Red.			
		White light.			Less than 520 mµ.			500 to 650 mµ.			Greater than 595 mµ.			
hrs.	*min.*	B	Y	R	B	Y	R	B	Y	R	B	Y	R	
0	0	12.4	2.7	7.7	12.4	2.7	7.7	12.4	2.7	7.7	12.4	2.7	7.7	
2	15	12.3	2.7	7.7										
2	18				12.2	2.7	7.7							
5	19	12.3	2.7	7.7										
5	21				12.5	2.7	7.3							
5	23							12.3	2.7	7.3				
5	25										13.2	2.7	6.7	
8	9	12.0	1.7	6.7										
8	11				12.0	1.7	6.5							
8	13							12.2	1.7	6.7				
8	15										12.3	1.7	5.2	
12	39				12.4	1.7	5.7							
12	42							12.2	1.7	5.7				
12	44										12.7	1.7	5.0	
26	52	11.8	1.7	4.7										
26	58				11.9	1.9	3.2							
27	3							12.5	1.7	4.7				
27	7										12.0	2.4	2.7	
48	15	13.0	2.7	4.5										
48	18				12.2	3.3	3.7							
48	19							10.3	4.3	4.1				
48	24										9.0	9.7	6.7	

B, Y, and R refer to blue, yellow, and red Lovibond units respectively.

3.1, red 13.6. The solution which was exposed to red light was the only one unaffected.

A 25 per cent solution of cod liver oil was exposed under the same conditions as the above extract with the results shown in

Table II. After 48 hours exposure there is only a small decrease in observed blue units; however, dilution curves on the solutions after exposure show that the destruction is very appreciable when the solutions are exposed to blue and white light. Fig. 5 indicates approximately 60 per cent destruction with blue light and 70 per cent destruction with white light. These results are similar to those obtained with the unsaponifiable extract.

The results of a second series of experiments with a chloroform solution of the unsaponifiable portion of cod liver oil are given in Table III. The conditions were similar to those given above, except that the filters were such that the first tube was in total

O UNFILTERED LIGHT.
● BLUE (LESS THAN 520Mμ).
● GREEN (FROM 500Mμ TO 650Mμ).
◉ RED (GREATER THAN 595Mμ).
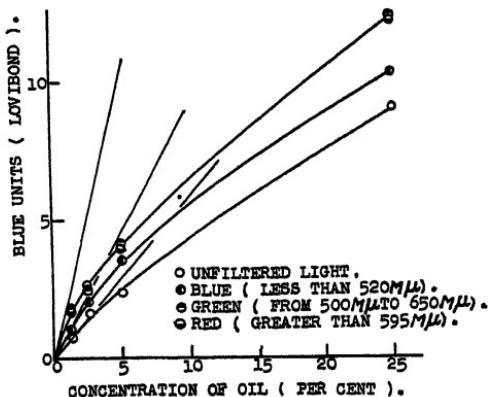
Fig. 5. Dilution curves showing the destruction of the chromogenic substance of cod liver oil when exposed to light of different wave-lengths.

darkness, the second had an amber filter transmitting light of wave-length greater than 515 $m\mu$, the third had a blue filter transmitting light of wave-length less than 515 $m\mu$, and the fourth was exposed to white light. The temperature of the room was 28–31°. The filter used with the second tube was very close in absorption to that of amber glass used for bottles and shows the protection afforded by removal of light with wave-length of less than 515 $m\mu$.

Three samples of cod liver oil were dissolved in chloroform to give solutions of approximately the same vitamin content and such that, estimating from the tangent to the dilution curve, each solution would have given approximately 50 blue units if the color

TABLE III.

Effect of Visible Light upon the Stability of Vitamin A in a Chloroform Solution of the Unsaponifiable Portion of Cod Liver Oil.

Duration of exposure.		Darkness.		Filter used.					
				Amber.		Blue.		None.	
				Greater than 515 $m\mu$.		Less than 515 $m\mu$.		White light.	
		B	Y	B	Y	B	Y	B	Y
hrs.	*min.*								
0	0	12.3	4.6	12.3	4.6	12.3	4.6	12.3	4.6
2	2	12.0	4.3						
2	4			12.1	4.2				
2	6					11.5	4.2		
2	8							11.2	4.0
5	7	11.5	4.7						
5	9			11.4	4.4				
5	12					9.6	3.2		
5	14							9.8	3.4
7	52	11.8	4.5						
7	56			11.8	4.1				
7	58					11.4	4.0		
8	5							9.5	3.4
12	25	12.0	3.9						
12	27			12.0	3.7				
12	30					9.9	3.7		
12	35							8.1	2.9
26	25	11.3	4.1						
26	36			11.1	3.9				
26	38					7.1	2.1		
26	42							4.1	1.0
48	4	10.7	4.0						
48	7			10.7	3.9				
48	10					3.3	0.7		
48	12							2.3	1.3

B and Y refer to blue and yellow Lovibond units respectively. The readings for red Lovibond units were negative except in the case of exposure of the solution to unfiltered white light for 48 hours and 12 minutes, which gave 0.4.

were a linear function of concentration. The solutions were exposed to the unfiltered light of a 200 watt tungsten lamp at 10 inches, and samples taken for colorimetric determination with antimony trichloride at intervals. The variation in observed blue units read at 30 seconds with duration of exposure is given in Fig. 6. It will be noticed that the observed blue shows a marked
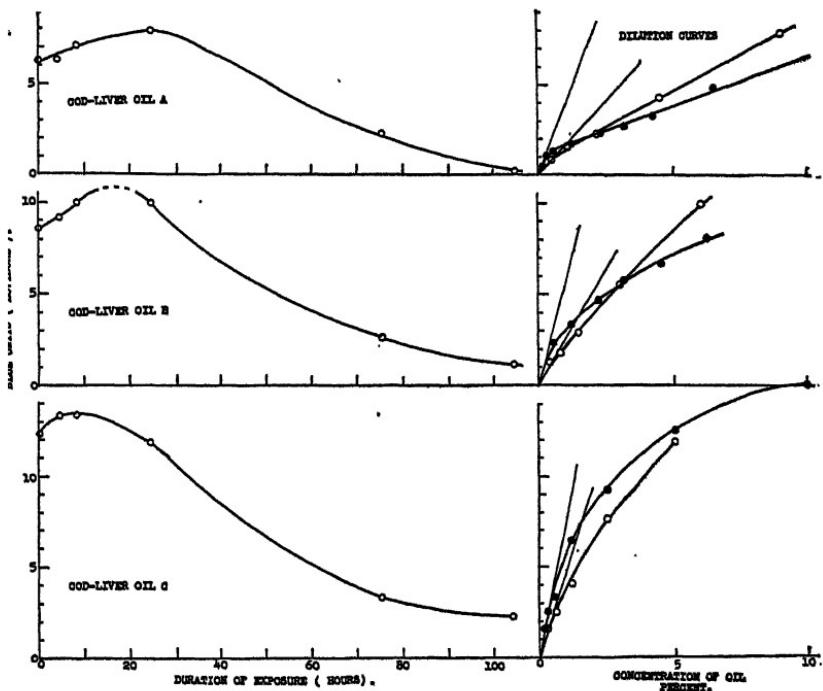
Fig. 6. The effect of exposure of cod liver oils to unfiltered light upon the observed blue coloration at 30 seconds in the antimony trichloride test, and the destruction of the chromogenic substance after 24 hours exposure as shown by dilution curves. In the dilution curves solid dots represent the original oil and circles the values after 24 hours exposure to light.

increase, followed by a decrease on exposure in each case. After 24 hours exposure to the light, dilution curves were run upon samples taken from each oil. The results are given in Fig. 6. Although the observed color is higher than the initial reading in Oils A and B and only slightly lower in Oil C, the tangents to the dilution curves show that the destruction has been greater than

50 per cent in each case.   The observed color is the resultant of the
various factors present and as the original solutions, which should
have read about 50 blue units if the color were a linear function of
concentration, only gave readings of 6.3, 8.6, and 12.3 respectively
for Oils A, B, and C, variations of a few units at this concentration
can have no significance.   The only significant values are those
in which the color is a linear function of concentration.

Hawk (12) in 1929 exposed samples of cod liver oil (uncovered)
to rain, snow, and sunlight.   After 79 hours of exposure during 2
weeks these samples gave a deeper blue color than like samples
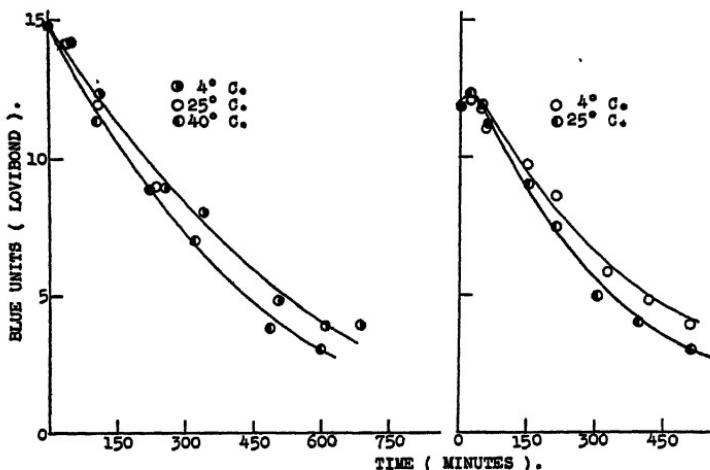
FIG. 7. The influence of the temperature during exposure upon the de-
struction of the chromogenic substance of cod liver oil by light.

kept in a clean, dry, dark closet or the original samples, which
would undoubtedly be the case with the conditions under which
he made the tests.   However if he had carefully run dilution
curves of the oils he would probably have found considerable
destruction of the chromogenic substance.   His experiments were
not sufficiently extensive to draw the conclusion that the antimony
trichloride test is not reliable for testing the vitamin A content of
cod liver oils.

The above experiments dealing with exposure of the chromo-
genic substance of cod liver oil to light were conducted at room

temperature which varied at different times from 24–31°. In order to determine what influence temperature might have upon the rate of destruction by light, series of experiments were performed upon the chloroform solution of the unsaponifiable fraction of a cod liver oil, from which the cholesterol had not been removed. The tubes exposed to the unfiltered light from a 200 watt tungsten lamp were maintained at 4°, 25°, and 40° by means of glass water baths and samples removed at intervals for testing with antimony trichloride. Fig. 7 gives the values obtained, corrected for the thermal expansion of the chloroform solution. It is observed
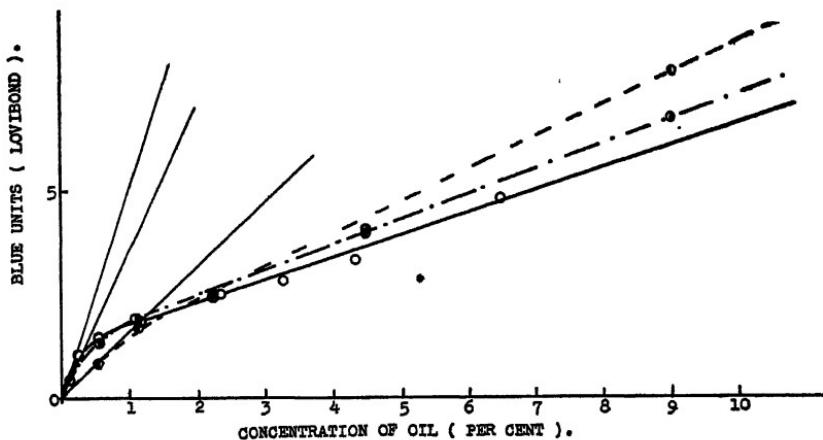
FIG. 8. The influence of oxygen on the destruction of the chromogenic substance of cod liver oil by light. O———O, dilution curve of the original oil before exposure to light. O—.—O, after 24 hours exposure to unfiltered light in an atmosphere of nitrogen. O---------O, after 24 hours exposure in an atmosphere of oxygen.

that the rate of destruction is only slightly increased by increasing the temperature of the solution during exposure to light.

It has been definitely shown by many workers that vitamin A is stable at relatively high temperatures in the absence of oxygen, but is rapidly destroyed when heated in the presence of oxygen. A test was made to determine whether oxygen would increase the rate of destruction of the chromogenic substance with light. Nitrogen was passed through a sample of cod liver oil for some time to sweep out any dissolved oxygen and saturate the oil with nitrogen; a second sample of the same oil was treated in a similar

nanner with oxygen to saturate the oil with the gas; the tubes
vere then tightly stoppered with corks coated with collodion,
eaving an atmosphere of nitrogen and oxygen above the oil in the
·espective tubes.   The oils were then exposed to white light as in
:he above experiments for 24 hours.   At the end of the period
:olorimetric dilution curves were made on the oils, the results of
which are given in Fig. 8.   From the graph it is observed by esti-
mating the tangents to the curves that there is more than twice
the destruction with oxygen present as in an atmosphere of nitro-
gen, the destruction being 67 per cent and 28 per cent respectively.
It will also be noticed that at 10 per cent a reverse order of color
intensity is found, the values being 6.7, 7.4, and 8.7.   However,
variations in so concentrated a solution which varies so widely
from a linear function, can have no significance.   As the observed
color of a solution is the result of the action of antimony trichloride
with all possible substances present in the oil, apparently the rate
of destruction of the blue chromogenic substance and of the sub-
stances which inhibit the blue formation or increase the rate of
fading of the blue are not equal. •

SUMMARY.

1. The effect of concentration of the antimony trichloride re-
agent upon the color produced with the chromogenic substance of
cod liver oil has been shown.

2. At low color values the variation of the observed color with
concentration of antimony trichloride is slight.

3. With high concentrations of oil the observed color at 30
seconds is dependent upon the light used for measurement of the
color.

4. The chromogenic substance of cod liver oil or vitamin A is
rapidly destroyed by light of wave-length less than 500 $m\mu$ and
practically unaffected by light of wave-length greater than 500 $m\mu$.

5. The presence of oxygen increases the rate of destruction of
vitamin A by light.

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THE TOXIC EFFECT OF FISH LIVER OILS, AND THE ACTION OF VITAMIN B.

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3 years ago in the biological assay of fish liver oils for vitamin A, it was noticed that relatively small amounts of some fish liver oils (less than 2 per cent of the diet) had a very deleterious effect, and a systematic study was initiated to determine what factor or factors of the oil were toxic or injurious.

Previously the toxic effects of cod liver oil had been noticed and efforts made to correlate the effect with hypervitaminosis of one of the fat-soluble vitamins, the excess of free fatty acids, etc. However the published results are very contradictory. Mouriquand and Michel (1) in 1922 reported a noxious influence of large doses of cod liver oil in scurvy, especially when the diet is lacking not only in vitamin C, but also in certain amino acids and salts. In 1925 Slagswold (2) noted poisoning among cattle in Sweden by cod liver oil, stiffness, listlessness, tremors, loss of appetite, convulsions, etc. being produced and death resulting in a few hours or days; while rodents thrived on the same oil. Takahashi described a toxic effect of biosterin with paralysis in the rear extremities when fed at 10,000 times the growth-promoting dose. His results were not duplicated in the work of Drummond, Channon, and Coward (3). Von Euler and Widell (4) found that when the dosage of vitamin D increased many times, rats on marmite and lemon juice, sufficient under normal conditions, died in about 4 months. Agduhr (5) in a series of articles (1926–1929) reported the toxic effect of cod liver oil on mice. He stated that the poisonous effects are greatly influenced by the composition of the basal diet and its content of vitamins B and C, as well as warm weather and sunshine. Also that the susceptibility varies very much in different individuals of the same species and different species of animals show variations. The white rat is only susceptible in small degree.

COD-LIVER OIL S
10% YEAST

k7-4 DAYS

COD-LIVER OIL S
18% YEAST

COD-LIVER OIL P₁
10% YEAST

COD-LIVER OIL P₁
18% YEAST

BODY WEIGHT (GRAMS)ᵒ.

438
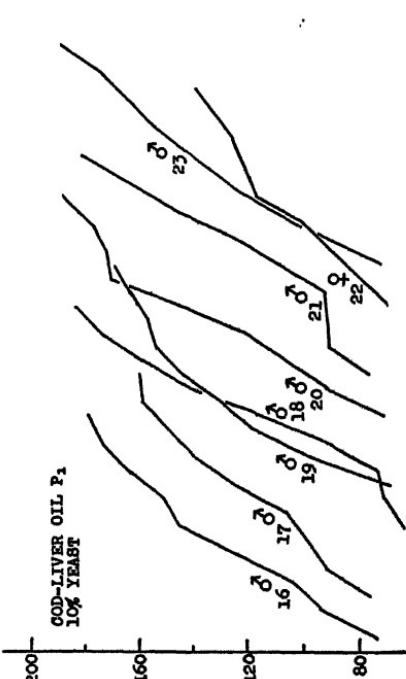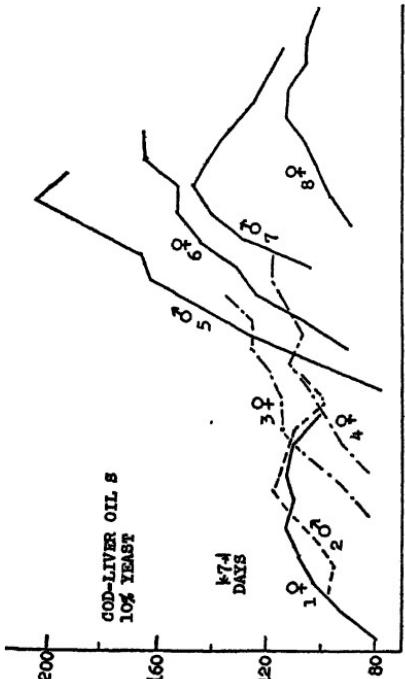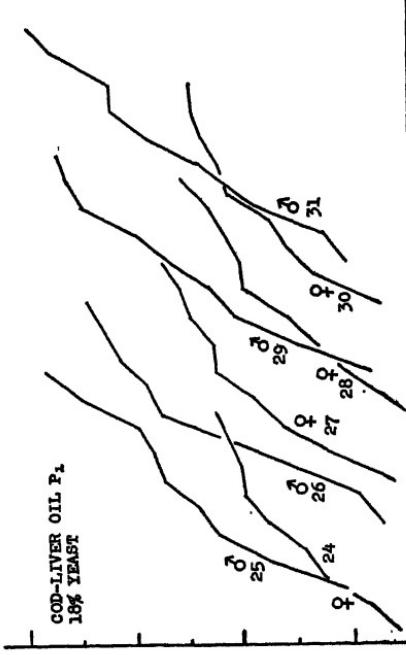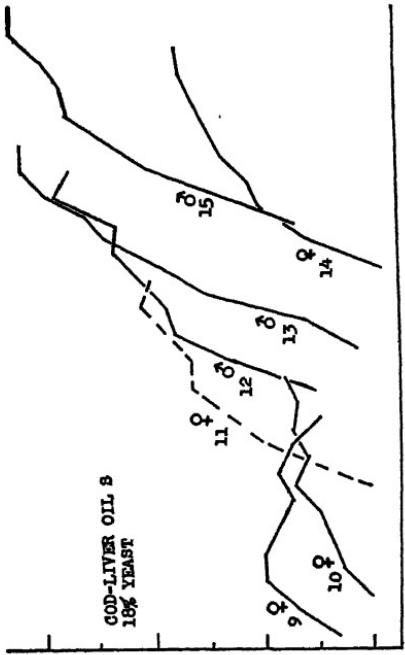
In testing oils for vitamin A a diet was used consisting of 65 per cent corn-starch, 20 per cent alcohol-extracted casein, 4 per cent Osborne and Mendel (6) salt mixture, 1 per cent sodium chloride, and 10 per cent dried yeast. To insure sufficient vitamin D the animals were irradiated 10 minutes three times a week at 25 inches with a 110 volt, Alpine sun, quartz mercury vapor lamp. The oil to be tested was fed over a wide range in amount and it was sometimes noticed that at the higher levels after 3 or 4 weeks there was a decrease in weight; the animals grew in length of body and apparent size but, being extremely thin, finally developed neurotic symptoms and paralysis, while at lower levels the animals grew at a normal rate. As the experimental animals on large doses of cod liver oil and a basal diet containing 10 per cent yeast showed symptoms so closely resembling vitamin B deficiency the yeast content was increased in some cases, and was immediately followed by rapid increase in weight. Also the apparent toxic effect of cod liver oils was not uniform for different oils and therefore must have been due to some variable substance in the oil. The first variable factors which suggest themselves are the two known fat-soluble vitamins, A and D.

Systematic feeding experiments were made with several fish liver oils known to vary considerably in vitamin content. Two basal diets were used; one was the 10 per cent basal diet described above, and the second was the same except that it contained 18 per cent yeast. The oil was fed separately in amounts varying up to approximately 0.2 gm. per day, after the usual depletion period for vitamin A. Fig. 1 shows a few of the characteristic feeding curves obtained with two cod liver oils. Oil P_1 was known to have approximately 5 times the vitamin A potency of Oil S. With Oil S on the diet containing 10 per cent yeast, animals receiving 0.0007 gm. of oil per day showed a very marked ophthalmia and other symptoms of vitamin A deficiency with slight

FIG. 1. Growth curves of rats on basal diets containing 10 per cent and 18 per cent yeast and receiving various amounts of cod liver Oils S and P_1. Rats 1, 2, 9, and 10 received 0.0007 gm. of oil per day; Rats 3, 4, and 11, 0.006 gm. per day; Rats 5, 6, 12, and 13, 0.052 gm. per day; and Rats 7, 8, 14, and 15, 0.207 gm. per day of Oil S. Rats 16, 17, 24, and 25 received 0.0017 gm. per day; Rats 18, 19, 26, and 27, 0.016 gm. per day; Rats 20, 21, 28, and 29, 0.05 gm. per day; and Rats 22, 23, 30, and 31, 0.198 gm. per day of Oil P_1.

growth; with larger amounts of the oil improvement in growth was obtained to almost normal growth at 0.052 gm. per day.   With 0.207 gm. of oil the animals were apparently normal for 3 to 4 weeks, after which they lost weight rapidly becoming thin and at the end of 8 weeks showing the picture of vitamin B deficiency. With the same levels of oil but on a diet containing 18 per cent yeast a similar vitamin A deficiency was observed at low levels. However when fed 0.207 gm. of oil the animals showed normal rate of growth.   Oil P_1 gave practically normal growth for all levels of oil from 0.0017 to 0.198 gm. per day on both 10 and 18 per cent yeast.

In order to make a comparison of the results on the several oils tested, where both male and female rats were used, the data were calculated to percentage of normal growth.   Normal for both male and female under the conditions in our laboratory was obtained by averaging a large number of each respective group; average normal growth over an 8 week period after depletion of vitamin A was found to be for males 13.1 gm. per week and for females 9.8 gm. per week.   The average of all rats on each level of oil was calculated and the results for four fish liver oils are plotted in Fig. 2.

The relative vitamin A potency of the four oils of Fig. 2 is cod liver Oil S 1.0, Oil L 3.0, Oil P_1 5.0, and ratfish liver oil 0.3; from the curves it is very obvious that the toxic effect is not proportional to the vitamin A content.   It is therefore not a hypervitaminosis due to excess of vitamin A nor a correlation between vitamins A and B, as Oil L which is 3 times as potent as Oil S showed some toxicity but considerably less than Oil S; Oil P_1, 5 times as potent, showed no toxicity at the maximum level fed, while the ratfish liver oil, only 0.3 as potent in vitamin A, showed at least as great a toxicity as Oil S.   With the ratfish liver oil it was impossible to obtain normal growth on a diet of 10 per cent yeast as the toxic properties of the oil were manifested long before the oil could be fed in amounts sufficient to satisfy the vitamin A requirements of the animal; however on 18 per cent yeast normal growth was readily obtained with each of the oils.

It will also be noticed, especially with Oil S and the ratfish liver oil, that at low levels of the oil in the region of restricted growth where a vitamin A unit would be obtained there is a very marked
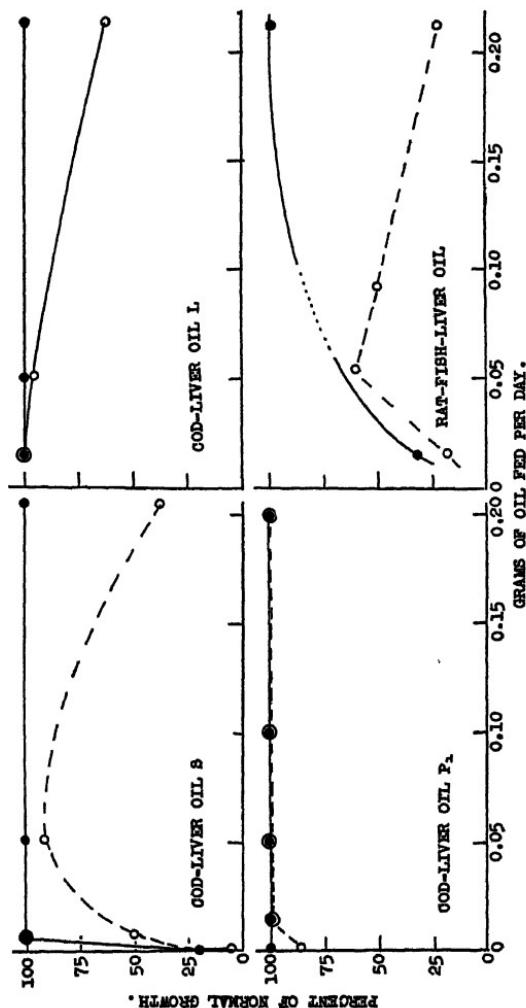
FIG. 2. Comparison of the growth obtained with four fish liver oils in various amounts by rats on basal diets containing 10 per cent and 18 per cent yeast. Circles represent 10 per cent yeast and solid dots 18 per cent yeast.

difference in response on 10 per cent and 18 per cent yeast, so that the unit would be quite different on the two diets. That 10 per cent yeast is ample for vitamin B requirements under certain conditions is shown by the fact that normal response can be obtained with the cod liver oils, especially with Oil P_1 which gives normal growth at all levels tried. Then what interpretation is to be placed upon the statement in the U. S. P. method for determination of vitamin A, "Use sufficient dried brewers' yeast to meet the vitamine B requirements of the animal?"

Many statements are to be found in literature indicating a similar difficulty of decreased growth with increased dosage of cod liver oil, as that of Schmidt-Nielsen (7) in discussing fish liver oils in which he states that a certain oil "proved to be sufficient for a satisfactory growth, when it was applied in daily doses of 1–2 mg. The same results were also obtained with the extracted liver oil and we were disposed to conclude from this result that the content of vitamin A in both samples corresponded to 1000 –500 units. This proved, however, to be quite erroneous. The extracted liver oil gives still more excellent growth, if it is applied in great dilutions e.g. in doses of 0.1 mg. a day, and the content of vitamin A is therefore in reality not 1000, but about 10,000 units." Drummond and Morton (8) in speaking of the biological assay state, "But even when the results for each group are averaged, a more serious type of discrepancy remains. This is shown clearly in the lower average rate of growth on 10 mg. of the oil J than on the dose of 7 mg. The same difficulty arises in the case of oil L." Their Oil J shows a growth of 4 gm. per week for a dose of 7 mg. and 2.6 gm. per week for a dose of 10 mg., results somewhat analogous to those we obtained with ratfish liver oil on 10 per cent yeast, where normal growth could not be induced. However, the results of Drummond et al. are for the short and variable period of 3 to 6 weeks, while all experiments in this laboratory are continued for 8 weeks.

The possibility of the yeast's containing a small amount of the growth-promoting factor measured as vitamin A was eliminated by running controls on both the 10 per cent and 18 per cent diets without added vitamin A. Ophthalmia appears at practically the same time and death occurs in the same period of time, as shown by characteristic curves in Fig. 3.
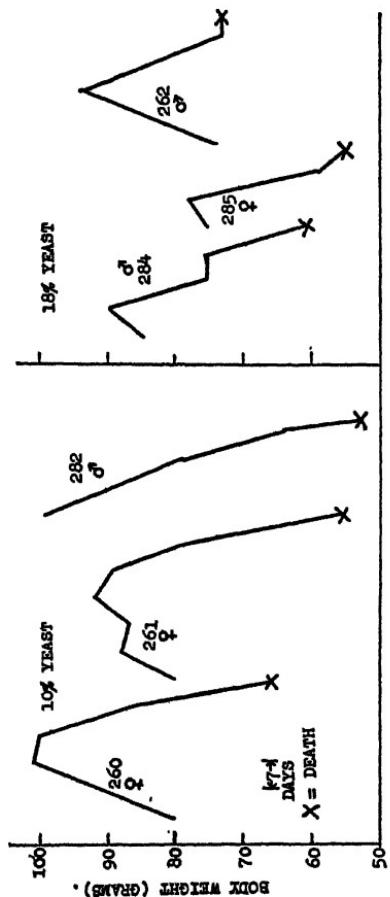
Fig. 3. Growth curves of rats on vitamin A free diets containing 10 per cent and 18 per cent yeast. Rat 260 died on the 34th day of the experimental period. Autopsy showed xerophthalmia, dilated bladder, and large pus pockets in tongue. Rat 261 died on the 42nd day of the experimental period. Autopsy showed vitamin A deficiency symptoms. Rat 282 died on the 23rd day of the experimental period. Autopsy showed vitamin A deficiency symptoms. Rat 284 died on the 30th day of the experimental period. Autopsy showed hemorrhagic lungs, pus pockets at the base of the tongue, apparent fusion of bladder and intestines, and xerophthalmia. Rat 285 died on the 19th day of the experimental period. Autopsy showed dilated bladder with hemorrhage at the base, pus pockets at the base of the tongue, and xerophthalmia. Rat 262 died on the 33rd day of the experimental period. Autopsy showed distended bladder, pus pockets in the roof of the mouth and in the base of the tongue, and xerophthalmia.

That the deleterious effect might be hypervitaminosis due to excess of vitamin D, was ruled out by feeding a series of rats 0.05 gm. of Oil S per day, which would give good growth (but which was the highest level of Oil S that would give good growth on a 10 per cent yeast diet) and by adding excess vitamin D in the form of activated ergosterol in amounts which would be equivalent to approximately 2 and 4 times the amount received with 0.2 gm. of Oil S per day, if the oil is an average oil as stated by the manufacturers. The animals showed normal growth with added ergosterol on both 10 and 18 per cent yeast. The literature on hypervitaminosis caused by high vitamin D content is somewhat contradictory but it seems the vitamin must be present in amounts equivalent to 10,000 to 100,000 times the curative dose to produce a deleterious effect. The amounts fed in the oils used in the above experiments showing toxicity could not have been of this magnitude.

After the above experiments had been completed and we were studying further the cause of the toxic effect, several papers appeared showing a similar effect. Harris and Moore (9) in 1928 studying hypervitaminosis noticed that when 15 per cent cod liver oil was substituted for 15 per cent arachis oil in a ration containing restricted allowances of vitamin B complex, a lower rate of growth was obtained and the animals had rough coats. Also in 1929 (10), they reported that the requirement for marmite is increased *pro rata* when the excess of cod liver oil concentrate is increased (vitamins A and D). The normal amount of marmite is inadequate with excessive dose of cod liver oil. They considered that probably an antagonistic effect is exerted between vitamin B complex and vitamin A; but they also state that the possible action of other unidentified substances is not excluded. Light, Miller, and Frey (11) in 1929 found that the acute symptoms of vitamin D overdosage can be counteracted at certain levels by liberal feedings of yeast.

In the above experiments of this paper the maximum level of cod liver oils fed was 0.2 gm. of oil per day which would average about 2 per cent of the diet. In testing food substances for vitamin B it has become common practice to add at least 2 per cent of cod liver oil to the diet to supply the required fat-soluble factors. To study the quantitative determination of vitamin B, Sherman and

Spohn (12) in 1923 used two basal diets, in the first of which vitamin A was furnished by 10 per cent butter fat and in the second by 8 per cent butter fat and 2 per cent cod liver oil; and they found in testing milk that it required 12 cc. when cod liver oil was added to the diet to give growth over an 8 week period approximating that given by 8 cc. without the cod liver oil. They state, "The reason for this difference is not apparent." Sherman and Axtmayer (13) in 1927 used 8 per cent butter fat and 2 per cent cod liver oil in studying the multiple nature of vitamin B. Also Hunt (14) in 1928 in testing the complex nature of vitamin B used 2 per cent cod liver oil. Croll and Mendel (15) in testing the vitamin B content of the maize kernel used 5 per cent cod liver oil. How different would be the results in testing for vitamin B in the yeast used, or any food stuff, with four different basal diets each containing 2 per cent of one of the four fish liver oils tested above (cod liver Oils S, L, P_1, or ratfish liver oil). At 2 per cent of the diet each of these oils would furnish ample vitamin A but each would give an entirely different result for vitamin B in any substance tested. So that vitamin B determinations with cod liver oil of unknown toxicity to furnish vitamin A cannot be compared.

Cod liver oil is not a simple mixture of neutral glycerides but contains some phospholipids as lecithin and consequently choline, as well as various sterols. Also in 1888 Gautier and Mourgues (16) found cod liver oils to contain several nitrogenous bases making up an average of 0.2 per cent of the oil. While the discoverers considered these bases leucomaines dissolved from the hepatic cells, the freshness of the livers used in preparation of the cod liver oil must greatly influence the bases or ptomaines dissolved by the oil. They found butylamine, isoamylamine, hexylamine, dihydrolutidine, asellin, morrhuin, and morrhuic acid. Isoamylamine constituting one-third of the total bases present, was found to be a very active poison, producing rigor, convulsions, and death. 4 mg. produced death in a greenfinch in 3 minutes. 3 mg. of asellin-HCl killed a greenfinch in 14 minutes. Hawk (17) confirmed the work of Gautier and Mourgues in 1907 and found 1.06 to 1.17 gm. of leucomaines per kilo of oil. He identified butylamine, amylamine, hexylamine, dihydrolutidine, and morrhuin, but found no asellin.
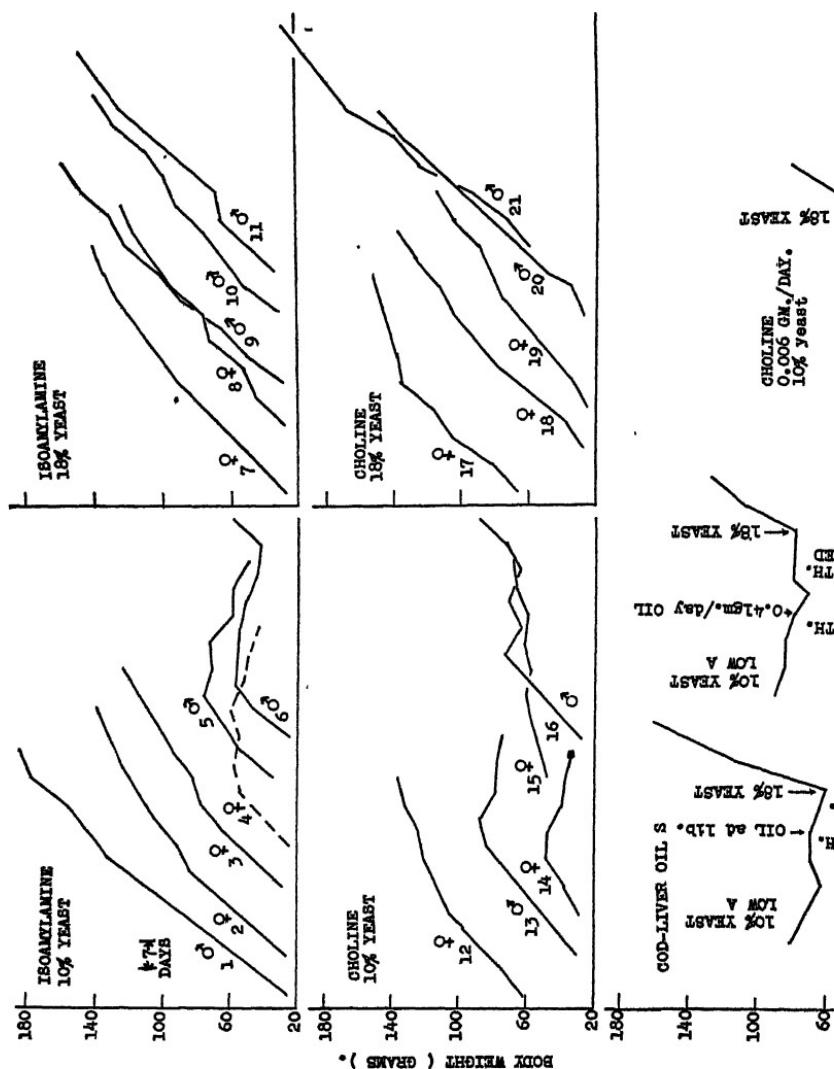
We demonstrated the poisonous effect of several basic amines including isoamylamine and choline-HCl when injected into the white rat, and then performed a series of experiments to test the influence of continued oral administration of small doses over a long period of time.

Young rats were weaned when 28 or 29 days of age and placed on .the 10 per cent and 18 per cent yeast, vitamin A-free diets, described above. The fat-soluble factors were furnished by giving 0.0165 gm. of cod liver oil P_1 per day. Isoamylamine was dissolved in oil and fed so as to give 0.00014 and 0.00056 gm. of the base per day over an 8 week period. If an oil as found by Gautier and Mourgues contains 0.2 per cent of bases one-third of which is isoamylamine, 0.2 gm. of the oil per day would be approximately equal to 0.00014 gm. of the base per day. Fig. 4 gives the growth curves obtained. On 0.00014 gm. per day variations were found on 10 per cent yeast, as in feeding oil at high levels. The animal showing loss of weight on 0.00014 gm. and both rats on 0.00056 gm. per day and 10 per cent yeast showed paralysis, convulsions, and typical symptoms of vitamin B deficiency during the last 2 weeks of the experimental period. All rats on 18 per cent yeast and receiving isoamylamine showed normal growth.

Fig. 4 also gives the results of experiments showing the effect of small amounts of choline over an extended period of time. The conditions were the same as in the case of isoamylamine and choline hydrochloride fed at levels of 0.015, 0.033, 0.066, and 0.098 gm. per day. On 10 per cent yeast the choline causes restricted growth, while on 18 per cent yeast growth is normal. On 0.098 gm. per day and 10 per cent yeast difficulty was experienced in having the animals eat all the material offered and consequently the dose was not quantitative, however in every other case the base was quantitatively consumed. At the end of the 6th week

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FIG. 4. Growth curves obtained by feeding rats basal diets containing 10 per cent and 18 per cent yeast supplemented with 0.0165 gm. per day of cod liver Oil P_1, and various amounts of toxic amines. Rats 1, 2, and 7 were controls and received no toxic substance. Rats 3, 4, 8, and 9 received 0.00014 gm. per day; Rats 5, 6, 10, and 11, 0.00056 gm. per day of isoamylamine. Rats 12 and 17 received 0.015 gm. per day; Rats 13 and 18, 0.033 gm. per day; Rats 14 and 19, 0.066 gm. per day; Rats 15, 16, 20, and 21, 0.098 gm. per day of choline hydrochloride.

on 0.066 gm. per day of choline-HCl the rat's hind extremities were paralyzed and it showed the symptoms of the terminal stages of vitamin B deficiency; it was then offered the 18 per cent diet and the administration of choline was continued as before. The symptoms of vitamin B deficiency immediately disappeared with rapid growth as shown in the lower right-hand corner of Fig. 4. The lower left-hand corner of Fig. 4 shows the recovery from toxemia produced by feeding high levels of cod liver Oil S on 10 per cent yeast by giving an 18 per cent yeast diet.

Further work which is nearing completion indicates that the effect of yeast is not specific for the two bases tested above. These results might explain some of the discrepancies and variations in both vitamin A and B determinations; as well as the effect of yeast or yeast extracts in counteracting an extremely high protein diet, as that described by Hassan and Drummond (18) (high protein diets are known to increase intestinal putrefaction with increased ptomaine formation), also the effect of yeast in low grade chronic infections.

SUMMARY.

1. Some cod liver oils produce symptoms similar to vitamin B deficiency when fed in relatively large amounts to animals on a diet containing as high as 10 per cent yeast.

2. The toxic effect of excess cod liver oil can be counteracted by feeding large amounts of yeast.

3. The toxic effect of large doses of some cod liver oils is not due to hypervitaminosis due to excess of vitamins A or D.

4. Continued small doses of isoamylamine in amounts which may be found in cod liver oil produce paralysis, convulsions, and lack of growth; the toxic effect may be prevented or cured by added yeast.

5. Continued small doses of choline also produce symptoms identical with vitamin B deficiency, which may be prevented or cured by added yeast.

6. The quantitative determination of vitamin A in cod liver oil is influenced by the amount of yeast in the basal diet, even when there is sufficient yeast to satisfy the vitamin B requirements under some conditions.

7. Determinations of vitamin B by methods of increase in rat

weight, where a cod liver oil of unknown toxicity is incorporated in the diet, cannot be compared.

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FURTHER STUDIES OF BLOOD CREATININE.

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Whether creatinine is present in normal blood, and accumulates after impairment of renal function, is a question of interest in connection with creatinine metabolism in general and with the origin of the creatinine of urine. In experiments previously reported (1) a direct approach to this problem was begun with the aid of several isolation methods. Creatinine was obtained, in the form of several of its derivatives, from normal blood of cattle. Much larger quantities were isolated from blood of nephritic patients, as well as from blood of dogs in which retention had been produced experimentally either by ligation of both ureters or by double nephrectomy. It is of further interest to know whether creatinine can be isolated from normal blood of more than one species, whether the larger yield is common to blood from all forms of retention, and whether the creatinine isolated is present in the blood as such or as a related compound.

The methods employed in extending the studies along the three lines mentioned are given in detail below, but for purposes of discussion the following outline will suffice. Blood filtrates are shaken with Lloyd's reagent—a hydrated aluminum silicate. The adsorbent is separated, and by means of a weak alkali a substance is recovered from it which reacts with alkaline picrate in a manner similar to creatinine. This substance, after evaporation in acid solution, is isolated as creatinine potassium picrate, from which other creatinine derivatives can be prepared.

Isolation Experiments.

In isolation experiments normal dog blood is preferable to beef blood, because it can be obtained without delay. At the same time the results are of greater interest because the dog is fre-

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quently used in studies of creatinine metabolism. Blood was
therefore obtained by bleeding normal dogs, which had fasted for
14 hours previously, from the carotid artery under brief ether anes-
thesia. Tungstic acid filtrates (1:10 dilution) were prepared at
once. Six lots were elaborated. From the Lloyd's reagent, with
which these filtrates were shaken, the amounts of apparent creati-
nine recorded in Column 2 of Table I were recovered. Calculated
per 100 cc. of blood, they would vary from 0.49 to 0.71 mg. That
this apparent creatinine was either true creatinine or a derivative
of it giving the same color reaction was indicated by the fact that
over 90 per cent of it could be precipitated from the combined lots

TABLE I.

Isolation Experiments on Blood of Normal Dogs.

Blood filtrate treated with Lloyd's reagent.	Creatinine released from Lloyd's reagent.		Per cent precipitated.
	Determined after evaporation.	Precipitated as picrate.	
liters	*mg.*	*mg.*	
7.72	3.86		
8.07	3.96		
9.04	6.43		
24.83	14.25	13.2	92.6
7.82	4.43		
11.65	6.90		
5.77	3.32		
25.24	14.65	13.4	91.5

as a picrate. Further details concerning this picrate are given
below.

In Table II the isolation results for blood from various types of
retention are given. The first sample is perhaps the most interest-
ing, because retention was not extreme. This specimen was ob-
tained from a patient with chronic cardiorenal disease, admitted
to the hospital because of myocardial insufficiency. A sufficient
quantity of blood for isolation purposes became available as the
result of venesection 9 days preceding death. The next two sam-
ples in Table II represent the familiar extreme retention of late
nephritis and prostatic obstruction; the remaining three, various

types of experimental retention. Blood from dogs in which deep x-ray treatment of the kidneys (2), or anastomosis of bladder and ileum, was the cause of retention was kindly furnished the writer by Dr. F. W. Hartman from experiments in progress. Both of the procedures produce a slowly developing retention, so that the time of obtaining blood was months after x-ray treatment or surgical operation and at the same time preceded development of a moribund state. The last sample in Table II represénts an acute type of nephritis. Uranium acetate, 1 mg. per kilo, was administered to the animal subcutaneously. The blood was obtained 8 days later.

The filtrates of these blood samples were treated with Lloyd's reagent as before. The amounts of apparent creatinine recovered

TABLE II.

Isolation Experiments on Blood from Cases of Retention.

Source of blood.	Creatinine per 100 cc. blood.		Per cent precipitated.
	Released from adsorbent.	Precipitated as picrate.	
	mg.	*mg.*	
Human nephritic, venesection...............	1.67	1.56	93.4
" " autopsy..................	19.5	17.2	88.2
Prostatic obstruction, autopsy...............	20.7	19.9	96.1
Dog, ileovesical anastomosis...............	3.8	3.67	96.6
Dog, x-ray nephritis.......................	5.0	4.82	96.4
Dog, uranium nephritis.....................	12.0	11.3	94.2

from the adsorbent are given, in mg. per 100 cc. of blood, in the second column in Table II. As in the case of normal blood, this apparent creatinine was almost entirely precipitated with picric acid and potassium chloride.

In order to identify the picrates obtained, the following experiments were carried out. A combined lot of double picrate obtained from experiments on normal bood, and containing 33.1 mg. of creatinine, was recrystallized three times. Picrate containing 31.4 mg. of creatinine remained. It was converted to creatinine zinc chloride in the manner previously described (1), with a yield of 90 per cent. For known creatinine potassium picrate the yield is 92 to 93 per cent. The creatinine zinc chloride was dissolved

in dilute hydrochloric acid. The solution contained 0.38 mg. of nitrogen per mg. of creatinine (6 microanalyses). Theory, 0.371 mg. Similar results were obtained in the case of the picrate from the third sample in Table II, which, in contrast with the preceding samples, represented extreme retention. In the remaining cases the criterion was that of obtaining picrates which could be recrystallized from saturated picric acid solution with very small losses, in the presence of a slight excess of potassium ion. They consisted of long pale yellow needles, the solution of which lost its creatinine on treatment with kaolin. These data appear to exclude the possibility that isolation yielded a substance unrelated to creatinine, simulating not only the color reaction of the latter, but forming a sparingly soluble picrate as well.

In numerous experiments it was found that the substance recovered from Lloyd's reagent, when determined colorimetrically, gave essentially the same values before and after evaporation with acid. Since the value after evaporation in turn corresponds with the creatinine content of the picrate subsequently separated, it is apparent that the isolation method can be changed into a simple colorimetric procedure. The substance in question is adsorbed from the filtrate, recovered from the adsorbent, and determined colorimetrically. Whether it is creatinine, an unknown derivative of creatinine giving the same color reaction, or a mixture of both, will be found quite immaterial in the question to be considered. The three possibilities are mentioned because the substance recovered from the adsorbent may be a creatinine compound, decomposed to free creatinine during evaporation.

Colorimetric Procedure.

In a 25 cc. test-tube place 20 cc. of tungstic or tungstomolybdic acid (3) filtrate. Add 1 cc. of 2 N (or saturated) oxalic acid solution, and 80 mg. of Lloyd's reagent (Eli Lilly and Company). Stopper the tube with a short cork fitting in such a way that the adsorbent will not stick between it and the tube. Place the tube horizontally in a shaking machine for 10 minutes. Centrifuge for 3 to 5 minutes. Decant and discard the supernatant liquid. Wash the inner surface of the tube with 3 to 5 cc. of water flowing from a pipette. Suspend the precipitate by gentle shaking. Centrifuge. Decant. Remove the hanging drop from the mouth of the

tube with filter paper. Add 50 mg. of magnesium oxide (U.S.P., heavy) and 6 cc. of water to the washed precipitate. Stopper the tube and shake it vigorously. Shake the mixture occasionally during the next 10 minutes. Centrifuge. With a pipette, transfer 5 cc. of the clear supernatant liquid to a dry test-tube.

The remaining steps are based upon the method of Folin and Wu (4). To the 5 cc. of unknown solution, add 2.5 cc. of alkaline picrate solution prepared by mixing 5 cc. of 2.5 N sodium hydroxide with 25 cc. of freshly prepared, saturated solution of picric acid. Add 5 cc. of the alkaline picrate solution to 10 cc. of an appropriate creatinine standard and make the color comparison after 10 minutes. During color development, centrifuge the unknown and decant it into a dry tube. This is routine procedure, since the cloudiness is so faint that it is usually not noticed until a poor match of colors is obtained. The matching has been perfect in a large series of estimations.

In calculating the results, empirical curves were used. These were prepared as follows: To 10 cc. portions of creatinine solutions containing 0.05 to 0.25 mg. of creatinine per 100 cc. (0.05 mg. intervals), were added 5 cc. portions of alkaline picrate solution. After 10 minutes the solution originally containing 0.15 mg. per 100 cc. was read against itself; it then served as a standard for each of the other determinations. The average readings from four series of determinations were plotted against the known concentrations. Similar curves to be used with standards containing 0.3, 0.6, and 1.2 mg. of creatinine per 100 cc. were prepared. The curve for the weakest standard, while quite regular, deviates a great deal from the one assumed in the usual calculation $\frac{S}{R} \times$ standard; the next two deviate progressively less, the last, scarcely at all.

The curve for the standard used yields the concentration of creatinine corresponding to the colorimetric reading. This concentration, in mg. per 100 cc., times 3, equals the result in mg. per 100 cc. of blood, since the 6 cc. of creatinine solution, of which a portion was compared with a standard, represent 2 cc. of blood (20 cc. of 1:10 filtrate).

The picric acid used was purified by way of sodium picrate, in the manner recently described by Benedict (5). When new

lots were purified, new sets of curves were prepared. Differences in the curves were definite, but proved to be negligible on account of the small calculation factor in the colorimetric procedure.

The strongest standard used, 1.2 mg. of creatinine per 100 cc., corresponds to only 3.6 mg. of creatinine per 100 cc. of blood. When the concentration is greater than this, the determination is diluted with a solution prepared by adding 1 volume of the alkaline picrate solution to 2 volumes of water. The result is multiplied accordingly.

The Lloyd's reagent and magnesium oxide may vary over 25 per cent in amount without altering the result, hence they may be measured. Measuring cups are conveniently made by fusing a piece of glass tubing completely, bending it at right angles at the point of fusion, and cutting off the two arms to serve respectively as handle and cup.

Experiments with Blood Filtrates Treated with Kaolin or Heated with Alkali.

Behre and Benedict (6) have shown that tungstic acid filtrates of normal blood contain as much apparent creatinine after being heated with alkali as before such treatment. Similarly, they found that treatment of picric acid filtrates of normal blood with kaolin did not alter the apparent creatinine content. Since heating with alkali destroyed added creatinine, and treatment with kaolin removed it, they conclude that the substance determined in current methods is not creatinine, and that creatinine is not present in blood in detectable quantities.

It has been very difficult to harmonize the results of isolation with the findings of Behre and Benedict without making unwarranted assumptions in connection with the methods involved in either case. The colorimetric procedure described above eliminates the evaporation with acid used in the actual isolation experiments, hence one is concerned only with changes which may be brought about by adsorbents.

In Table III some of the results are shown. With the exception of the last four specimens, which represent early retention, all of the blood samples were drawn from normal dogs during the postabsorptive period. The values given by the above colorimetric procedure are designated as creatinine obtained from blood fil-

trates; those given by the original Folin method (7) as "creatinine" present in blood filtrates.

Columns 4 to 6 of Table III present a repetition of the experiments of Behre and Benedict, with essentially identical results.

TABLE III.

Showing That Creatinine or a Derivative of It Is Regularly Obtained from Blood Filtrates, but Cannot Be Demonstrated in Original Filtrates.

All figures represent mg. per 100 cc. of blood.

Creatinine obtained from blood filtrates.			"Creatinine" present in picric acid filtrates. Folin method (7).		
Tungstic acid filtrate. (1)	Picric acid filtrate (7).		Before kaolin. (4)	After kaolin. (5)	Difference. (6)
	Before kaolin. (2)	After kaolin. (3)			
0.35	0.30	0.04	1.33	1.44	+0.11
0.35	0.31	0.08	1.11	1.17	+0.06
0.44	0.38	0.09	1.13	1.25	+0.12
0.52	0.36	0.10	1.25	1.25	0.00
0.53	0.42	0.09	1.20	1.23	+0.03
Average. 0.44	0.35	0.08	1.20	1.27	+0.07
0.53	0.45	0.04	1.25	1.36	+0.11
0.57	0.39	0.04	1.35	1.37	+0.02
0.57	0.40	0.04	1.39	1.41	+0.02
0.66	0.46	0.10	1.25	1.27	+0.02
0.83	0.61	0.09	1.33	1.27	−0.06
Average. 0.63	0.46	0.06	1.31	1.33	+0.02
1.15*	0.75	0.12	2.27	2.17	−0.10
1.32*	0.84	0.14	1.91	1.88	−0.03
1.36*	0.90	0.19	2.59	2.34	−0.25
1.62*	1.05	0.17	2.67	2.27	−0.40
Average. 1.36	0.88	0.16	2.36	2.16	−0.20

* Blood specimens from cases of early retention.

In these experiments the Folin picric acid filtrate is shaken with kaolin, and the chromogenic substance present before and after kaolin treatment is determined by adding alkali to the filtrate and comparing the color obtained in 10 minutes with that developed in picric acid solutions of creatinine similarly treated. Column 6

in Table III is purely mathematical.   Certainly there is no evidence that kaolin removes a chromogenic substance.

It should be mentioned that in the preceding studies (1) there was a decrease of apparent creatinine in picric acid filtrates of normal blood after kaolin treatment.   This result is frequently observed if kaolin extracted by boiling with 20 per cent hydrochloric acid is used.   The present results are obtained with kaolin washed at room temperature with dilute (1:5) hydrochloric acid. This kaolin was entirely satisfactory for the removal of creatinine added to blood and recovered in the filtrate.

Now if the colorimetric procedure outlined above is used, the values in Columns 1 to 3 of Table III are found.   Creatinine is regularly obtained from tungstic acid filtrates of normal blood. Parallel amounts are obtained from picric acid filtrates of the same blood.   Considerably larger quantities appear early in retention. But if the picric acid filtrate is first treated with kaolin, no noteworthy amounts of creatinine or other chromogenic substance are obtained (Column 3).   In other words, none of the chromogenic substances present in such filtrates after kaolin treatment are removed by Lloyd's reagent and released again.   If these include all the chromogenic substances present (see Columns 4 and 5), there should be none obtained from the filtrate before kaolin treatment, which is contrary to the observations (Column 2).   The substance obtained and giving the values in Column 2, whether creatinine or a derivative of it, must have been formed in the process of adsorption by and recovery from Lloyd's reagent.

For tungstic acid filtrates the results are even more clear cut. The amounts of creatinine obtained by the above colorimetric procedure (and by actual isolation) are larger than in the case of picric acid filtrates, as shown in Columns 1 and 2 of Table III. But if the tungstic acid filtrates are first heated with alkali, neutralized again, acidified with oxalic acid, and used, only traces of chromogenic substance will be found that are adsorbed by Lloyd's reagent and released again.   These amount to only 0.05 mg. per 100 cc. of blood in terms of creatinine.

The substance recovered from Lloyd's reagent is chromogenic, removable from its solutions by kaolin, and destructible by heating with alkali.   It has all of these properties of creatinine before evaporation with acid and precipitation as a picrate, and the above

ındirect findings indicate that it has the two properties last mentioned, in the original filtrate. The specific difficulty in harmonizing the contradictory results is then the assumption that the chromogenic property of the substance released from Lloyd's reagent has been acquired in the process of adsorption and recovery from the adsorbent. If this assumption is not made, one excludes creatinine from blood, and admits the presence of a substance identical in properties.

Reasonably accurate determinations of the small amounts in Columns 1 to 3 of Table III were made possible because the substance determined is released into a much smaller volume than that from which it is adsorbed. The amount removed from 20 cc. of filtrate is released again into 6 cc. of solution. In the case of the tungstic acid filtrate this amounts to concentrating a 1:10 dilution of blood to a 1:3 dilution (calculation factor = 3); in the case of the picric acid filtrate a 1:5 dilution becomes a 2:3 dilution (calculation factor = 1.5). The colors actually matched in Column 2 were usually deeper than those in Columns 4 and 5. Those in the upper part of Column 3 were still 7 mm. above the reading of sodium picrate itself, which is the zero point of the empirical curve for the weakest standard.

Kaolin treatment consisted of shaking 37.5 cc. of the picric acid filtrate with 3 gm. of kaolin, for 10 minutes. The proportions are the same as those used by previous workers (6, 8). The kaolin was removed by centrifuging. Sufficient supernatant liquid was obtained in this way for the determinations in Columns 3 and 5. After adding the alkali, in determinations by the Folin method (Columns 4 and 5) both untreated and treated filtrates were centrifuged during color development, the former to remove a trace of ferric hydroxide, the latter to remove a haze which is often present when kaolin-treated picric acid solutions are made alkaline.

In treating tungstic acid filtrates with alkali, 20 cc. of filtrate and 5 cc. of 2.5 N sodium hydroxide were heated for 1 hour in boiling water (6). The solution was cooled. 1 cc. of concentrated hydrochloric acid approximately neutralizes it. Acidification with oxalic acid, addition of Lloyd's reagent, and further treatment followed as described in the colorimetric procedure.

Experiments with Separate Filtrates of Corpuscles and Plasma.

Whether the creatinine obtained from blood originates primarily from either corpuscles or plasma was regarded as a question of interest, because a very uneven distribution could be regarded as evidence that this creatinine was originally a radical of a more complex compound characteristic of either cells or plasma.

The results of separate analyses of corpuscles and plasma by the colorimetric procedure described above are recorded in Table IV.

TABLE IV.

Comparison of Amounts of Creatinine Obtained from Corpuscles and Plasma.

Sample No.	Creatinine obtained per 100 cc.		
	Whole blood.	Plasma.	Corpuscles.
	mg.	*mg.*	*mg.*
1	0.39		0.32
2	0.53		0.47
3	0.56		0.49
4	0.58		0.55
5	0.58		0.62
Average..........	0.53		0.49
6	0.39	0.39	0.36
7	0.39	0.47	0.45
8	0.44	0.49	0.42
9	0.45	0.49	0.39
10	0.45	0.52	0.37
11	0.49	0.45	0.35
Average..........	0.43	0.47	0.39

The specimens of blood were drawn from dogs during the post-absorptive period. After withdrawal of samples for preparation of the usual filtrate of whole blood, the remaining portions were centrifuged until the corpuscle volume became constant. The plasma was then separated as completely as possible. In preparing filtrates, the proportions of sample, water, 10 per cent sodium tungstate, and $\frac{2}{3}$ N sulfuric acid used were; in the case of plasma 1:8:0.5:0.5; in the case of corpuscles, 1:5:2:2. The results indicate that the amounts of creatinine obtained from corpuscles and plasma are approximately equal.

The constant finding in the previous and present isolation experiments is that when blood filtrates are shaken with Lloyd's reagent very nearly all of the chromogenic substance subsequently recovered from the adsorbent can be isolated as creatinine in the form of its derivatives. This finding holds whether the alkali used in recovering the substance is hydrated lead oxide, as in previous experiments, or magnesium oxide, as in the present ones. It also holds when blood of normal dogs is used instead of beef blood, and when different types of retention are studied.

The above colorimetric procedure, based upon this finding, only involves shaking of blood filtrates with Lloyd's reagent, and shaking of the separated adsorbent with magnesium oxide suspension, both procedures being carried out at room temperature. Creatine added to blood does not affect the results in the least. Nevertheless, the creatinine or creatinine derivative obtained in this way appears to be formed from a non-chromogenic compound, since it is adsorbed by kaolin, yet cannot be demonstrated "by difference" in determinations carried out on the filtrates before and after kaolin treatment.

The view that the creatinine or creatinine derivative obtained from normal blood is formed, rather than that the unexpected isolation is accounted for by errors in detecting such small quantities by difference in the presence of a large amount of other chromogenic substances (1) has grown primarily out of the data recorded in Table III. If one omits the last two samples, the amounts of creatinine or creatinine derivative obtained from blood filtrates vary from 0.35 to 1.32 mg. per 100 cc. of blood in the case of tungstic acid filtrates, and from 0.30 to 0.84 mg. when picric acid filtrates are used, without noteworthy changes in the results obtained by difference in Column 6. It is impossible to assume that the larger quantities would have escaped detection by difference, and if these require a different explanation, this explanation becomes applicable to all of the smaller quantities. The failure to recover creatinine from kaolin-treated filtrates (Column 3) also indicates that kaolin removes the substance about as completely as Lloyd's reagent does. Hence there should be no discrepancies arising from incomplete removal of creatinine by kaolin.

For obvious reasons no actual isolation experiments on normal human blood have been carried out. Examination of sixteen samples of presumably normal human blood by the colorimetric procedure gave values of 0.4 to 0.6 mg. per 100 cc. of blood in terms of creatinine. The substance obtained from human blood is also destroyed by alkali and adsorbed by kaolin. It seems probable therefore that a substance other than creatine, capable of yielding creatinine, is also present in human blood. In a much larger series of blood samples drawn from normal dogs the range of values was somewhat greater, the large majority of the results falling between 0.4 mg. and 0.7 mg. per 100 cc. The colorimetric procedure described is however not offered as an additional analytical method. Since the substance yielding creatinine is not known, one can predict nothing as to the completeness or regularity of its recovery, although any given blood sample yields good duplicate results. The procedure has however been of value in that it gives essentially the same results as actual isolation, which is too tedious for detailed studies and often inapplicable.

Lloyd's reagent gives a very strong "oxidase" reaction. When added to alcoholic solutions of benzidine or guaiacum, or preferably to water suspensions prepared from such alcoholic solutions, the mixture becomes deep blue almost instantly. This catalysis of oxidation by various clays and fullers' earths has been previously noted by other observers, and other reactions including polymerization, isomerization, and hydrolysis are known to be brought about by these substances. The elimination of all procedures other than the use of adsorbents therefore does not exclude the possibility of altering a compound while separating it from other substances.

Magnesium oxide releases the chromogenic substance from Lloyd's reagent somewhat more completely than hydrated lead oxide. Unfortunately it also releases larger amounts of substances which are either of a peptone nature or are hydrolyzed to peptone during evaporation with acid. It was therefore necessary to introduce an additional step for the separaton of creatinine and peptone, into the isolation procedure. Peptone interferes with the precipitation of creatinine picrates.

The acid used in the isolation procedure does not appear to be a determining factor in the result. In the first isolation procedure

previously published, only one evaporation in acetic acid solution is carried out. The picrates obtained precipitated at once and corresponded with creatinine potassium picrate in creatinine ·content. If acetic acid is substituted for hydrochloric acid in the present procedure, crystalline picrates of the chromogenic substance are obtained quite as readily from retention blood as when the stronger acid is used. The evaporation with hydrochloric acid and lead was used because with this the creatinine values before and after evaporation were the most regular.

All forms of retention investigated have yielded the common finding that the amounts of creatinine isolated from the blood are very much larger than those obtained from normal blood. Whether retention is caused by nephritis, or obstruction, or whether it is produced by double nephrectomy, bilateral ligation of the ureters, deep x-ray treatment of the kidneys, reabsorption of urine, or acute uranium poisoning, appears to be immaterial. The result is not to be ascribed to a moribund condition, for this was avoided in many of the isolation experiments, and with the colorimetric procedure it was found that the accumulation of creatinine-yielding substance in the experimental types of retention is progressive from the beginning of the experiment to the end.

The findings in the present study are in agreement with the view of Behre and Benedict that creatinine is not present in blood in detectable quantities. Whether the creatinine-yielding substance in normal blood is a precursor of urine creatinine cannot be stated. Its accumulation in early retention suggests, but by no means establishes, this. Parallel studies of blood and urine in early retention and studies of blood from the renal artery and vein have not as yet been made.

CONCLUSIONS.

1. Blood of normal dogs contains a substance, other than creatine, yielding creatinine in isolation experiments. A similar substance is present in human blood. Approximately equal amounts are present in corpuscles and plasma.

2. Impairment of renal function results in an accumulation of one or more substances other than creatine, yielding creatinine.

3. Evidence is presented that the creatinine-yielding substance in normal blood, while not creatine, is not creatinine itself.

Isolation Procedure.

To 5 liters of tungstic acid filtrate, add 250 cc. of 2 N oxalic acid solution and 20 gm. of Lloyd's reagent. Shake the suspension for 10 minutes, then fill four 250 cc. wide mouthed centrifuge bottles with it. Centrifuge, decant, and repeat the process until all of the adsorbent has been collected. Add 185 cc. of water to each bottle, stopper, shake well, centrifuge, and decant. To each bottle, add 3 gm. of magnesium oxide and 200 cc. of water, in the order named. Stopper and shake vigorously. Transfer all the suspensions to a 2 liter beaker, a total of 1500 cc. of water being used for suspending and transferring. Allow to stand 10 minutes with frequent stirring. Centrifuge. Decant. Measure the decanted liquid and transfer it to a 1800 cc. Pyrex evaporating dish. Acidify with 3 cc. of concentrated hydrochloric acid. Add a pinch of granulated lead. Evaporate to dryness on the steam bath. With successive 10 cc. portions of water, extract the residue; filter each portion through the same filter, and wash to a total volume of 50 cc. 0.5 cc. is taken for analysis. The remainder is evaporated. This is Residue A.

Residues A are combined by solution and evaporation until 12 to 15 mg. of creatinine are available. Dissolve the combined residues in 50 cc. of water. Neutralize to litmus with 10 per cent sodium hydroxide. Filter from the lead precipitate, which carries down various impurities. Catch the filtrate in a graduate and wash to a total volume of 98 cc. Add 10 cc. of tungstomolybdate (3) solution, and 12 cc. of 0.67 N sulfuric acid. Filter after 20 to 30 minutes. Measure the filtrate, and transfer it to a 250 cc. centrifuge bottle, adding rinsings. Add 10 cc. of 2 N oxalic acid solution and 3 gm. of Lloyd's reagent. Stopper. Shake gently for 10 minutes. Centrifuge. Decant. Wash the Lloyd's reagent by suspending it in 100 cc. of water to which 2 cc. of 2 N oxalic acid have been added. Centrifuge. Decant. Add to the precipitate 2 gm. of magnesium oxide and 200 cc. of water (Suspension *B*). Stopper. Shake vigorously. Allow the suspension to stand for 10 minutes with occasional shaking. Centrifuge. Decant the supernatant liquid into a graduate. After measuring, transfer the decanted liquid to a small glass evaporating dish, acidify it with hydrochloric acid, and evaporate to dryness on a steam bath.

Mark a 25 cc. Pyrex test-tube at 10 cc. Dissolve and transfer the above residue to it with successive 2 cc. portions of water. Neutralize with 2.5 N sodium hydroxide, until a precipitate (aluminum hydroxide) remains on shaking. Add water to the 10 cc. mark. Add 300 mg. of dry, purified picric acid. Heat to solution. Cool under the tap to about 15°. An emulsion forms. Centrifuge at once, and decant the supernatant liquid into another Pyrex tube. To the decanted liquid add 0.2 cc. of 10 per cent potassium chloride solution. Set both tubes aside for several days. The creatinine potassium picrate will now have crystallized out. To recover any that was carried down in centrifuging the emulsion, decant the supernatant liquid, after centrifuging, upon the oily residue in the first tube. Heat to solution, cool under the tap, centrifuge, and decant the solution upon the crystals in the second tube. Allow to stand for several days. Then centrifuge and decant. Add about 9 cc. of 1.2 per cent picric acid solution to the crystals. Heat to solution. Add picric acid solution to the 10 cc. mark. Mix. Determine the creatinine content of the hot solution.

Calculation.—The amount of creatinine in the combined Residues A is obtained as follows: To the amount in the crystals, add 0.4 mg. to correct for solubility. Multiply this sum by $\dfrac{120}{a} \times \dfrac{200}{b} \times \dfrac{100}{91}$, a being the number of cc. of filtrate from the tungsto-molybdic acid precipitation, and b the number of cc. of liquid decanted after centrifuging Suspension B.

Notes.—In order to exclude unexpected changes, the creatinine present before and after each step in its isolation was determined in samples equal to about 1 per cent of the total volume. Where the volume is small, the sample is taken with a 0.1 or 0.2 cc. pipette, calibrated to contain. The sample is discharged into 9.9 or 9.8 cc. of water in a test-tube, and the pipette is rinsed by drawing the solution into it several times. 5 cc. of alkaline picrate solution are added, and the determination is made according to Folin and Wu (4).

Solutions similar to those of Residues A can be made from 6 cc. of a 10 per cent solution of bactopeptone (Digestive Ferments Company), 14 cc. of creatinine standard (1 mg. per cc. of 0.1 N

hydrochloric acid), and water to make 98 cc. They are convenient for preliminary trials of the procedure. The small empirical correction given above $\left(\dfrac{100}{91}\right)$ is also the one observed with such solutions. The amount of peptone is arranged so that the amount of nitrogen precipitated by tungstomolybdic acid is about the same as in the case of residues from normal blood containing a total of 14 mg. of creatinine.

After the first precipitation, each successive recrystallization of the double picrate from 10 cc. portions of 1.2 per cent picric acid solution loses only 0.2 mg. of creatinine, if 0.05 cc. of 10 per cent potassium chloride is added each time. The product changes from small brownish crystals to rapidly forming, pale yellow needles.

The amounts given in the isolation procedure are those for normal blood. When blood from cases of retention is used, all amounts and volumes are greatly reduced in the steps preceding the purification of Residue A. If no tungstomolybdate precipitate is obtained in purifying a residue from blood from cases of retention, sufficient purification will usually be accomplished by simply proceeding with the second adsorption on, and recovery from, Lloyd's reagent. The volumes can however be altered so that the tungstomolybdic acid precipitation is carried out in a smaller volume, and the resulting filtrate diluted before proceeding with the second adsorption.

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THE CALCIUM CONTENT OF STRIATED MUSCLE OF RACHITIC ANIMALS.

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Considerable work has been done on the calcium content of the skeleton of rachitic animals with no regard to the changes in the calcium content in the muscles of animals suffering from rickets. Although the percentage of calcium in muscle is small, it is highly important from a functional standpoint as evidenced by the rapid onset of tetany when the calcium of the blood falls below 3 to 4 mg. per cent.

The object of this study is to determine whether the calcium content of striated muscle of the rachitic rat varies from the calcium content of the striated muscle of the normal rat. Seventeen rachitic and ten normal rats were used in this study: The rachitic rats were fed on the low phosphorus-high calcium diet for 63 days. The diet is composed as follows: flour (high patent, low ash) 30 per cent, glucose 40 per cent, ash-free casein 17 per cent, prepared as described by Burr and Burr (1), Crisco 5.5 per cent, $CaCO_3$ 2 per cent, spinach (dry) 2 per cent, brewers' yeast 2 per cent, NaCl 1.5 per cent. The normal rats received the same diet plus 1 per cent NaH_2PO_4 and 1 per cent Na_2HPO_4.

EXPERIMENTAL.

The muscle of each rat was carefully dissected off, the fat carefuly removed, and the muscle then cut into fine bits and dried in an oven at 95° for 36 hours. The sample of muscle was then pulverized in a mortar, washed twice with alcohol, and finally with ether, thus removing fat from the muscle. (The analysis of the alcohol and ether wash solution gave negative results when analyzed for calcium.) The wash solution can be quickly separated

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from the sample by centrifuging. After washing with ether the sample was placed in an oven at 75° for ½ hour and then kept in a desiccator overnight. This dry sample was then weighed into an ignited tared silica dish and ignited in a muffle furnace at 400° to a white ash, usually requiring 24 to 36 hours. The ash was then dissolved in the minimum quantity of 10 per cent HCl and transferred quantitatively to a 100 cc. volumetric flask, a drop of alizarin added, and NH₄OH added until neutral or slightly alkaline, and then dilute HCl added until neutral or just acid. Now 5

TABLE I.

Calcium Content in Dry Striated Muscle of Rachitic and Normal Rats.

Rat No.	Sex.	Diagnosis.	Average weight of Ca per 100 gm. tissue.	Rat No.	Sex.	Diagnosis.	Average weight of Ca per 100 gm. tissue.
			mg.				*mg.*
730	♂	Rickets.	43.2	53	♀	Rickets.	44.5
731	♂	"	42.7	55	♀	"	44.8
732	♂	"	43.0	56	♀	"	42.2
733	♂	"	45.9	15	♂	Normal.	72.3
734	♀	"	43.5	16	·♀	"	83 5
735	♀	"	42.6	17	♀	"	67.2
736	♂	"	39.9	18	♀	"	83.4
737	♂	"	37.7	21	♂	"	92.1
738	♂	"	41.7	22	♂	"	73.6
739	♀	"	38.0	717	♂	"	62.2
740	♂	"	35.0	718	♂	"	56.8
50	♂	"	45.2	719	♂	"	68.7
51	♀	"	42.0	720	♂	"	78.6
52	♂	"	40.0				

drops of concentrated HCl (sp. gr. 1.20) were added and the volume made up to 100 cc. Aliquots of 20 cc. were transferred to 50 cc. centrifuge tubes and the calcium determined by McCrudden's method (2) as follows: 1 cc. of 2.5 per cent oxalic acid was run into the 20 cc. aliquot and then with stirring 1 cc. of 20 per cent sodium acetate was added. It was allowed to stand overnight. The precipitate of calcium oxalate was centrifuged and washed twice with 0.5 per cent ammonium oxalate solution and twice with distilled water to free from chlorides and oxalic acid. The water

was carefully decanted from the calcium oxalate precipitate and 5 cc. of normal H_2SO_4 added. It was heated to 75° and titrated at this tempeature with 0.01 N $KMnO_4$. 1 cc. of 0.01 N $KMnO_4$ is equivalent to 0.2 mg. of calcium. The calcium oxalate may also be transferred to an ashless filter paper, dried, and ignited to constant weight, and weighed as calcium oxide. This gravimetric method was used as a check and very close results were obtained. The average amount of calcium in mg. per 100 gm. of muscle tissue by the titration method is 41.6 and the average by the gravimetric method is 42.1.

Analyses were run on two samples from each rat. The average minimum weight of samples was about 2.5 gm., and the average maximum weight was about 5.0 gm. Two checks were run on each sample. This was made possible by making the volume up to 100 cc. and taking aliquots as described in the procedure. By this method four analyses were made on the muscles of each rat. Only the average figures for the analyses are given in Table I. Preliminary analyses were made on a sample of mule muscle to check the constancy of the results by this method. In over 85 analyses made by the titration method, the results checked within less than ± 0.1 per cent variation. Gravimetric determinations were also made on the same sample, with an average variation of less than 0.4 per cent.

SUMMARY.

The analyses of the striated muscles of rachitic rats show definite and marked decrease in calcium content. The average calcium content for the muscles of all the rachitic rats is 41.6 mg. per 100 gm. of dry muscle. The average calcium content for the muscle of normal rats is 74.0 mg. per 100 gm. of dry muscle, or 56 per cent higher than the calcium in rachitic rats.

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THE HYDROGENATION OF METHYLPHENYL AND METHYLBENZYL CARBINOLS.

WITH A NOTE ON THE REDUCTION OF PHENYLATED CARBINOLS.

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It was reported in a previous paper[1] that ethylphenyl, ethylbenzyl, and ethylphenethyl carbinols on hydrogenation yielded cyclohexyl carbinols with the same sign of optical rotation. Isopropylphenyl carbinol, however, on hydrogenation suffered a change in sign. This last was attributed to irregularities in the isopropyl series. But it was now found that methylphenyl carbinol also undergoes a change in sign, that it behaves like the isopropyl carbinol rather than like the ethylphenyl carbinol to which it seems more closely related. Methylbenzyl carbinol, however, yielded methylhexahydrobenzyl carbinol of the same sign of rotation. It follows therefore that, if the rule of Levene and Haller can be applied to cyclohexyl compounds, then dextro-methylphenyl and levo-ethylphenyl carbinols are configurationally related.

$$
\begin{array}{ccc}
\text{CH}_3 & & \text{CH}_3 \\
| & & | \\
\text{CHOH} & \longrightarrow & \text{HOCH} \\
| & & | \\
\text{C}_6\text{H}_5 & & \text{C}_6\text{H}_{11} \\
\text{Dextro.} & & \text{Levo.} \\
\updownarrow & & \updownarrow \\
\text{C}_2\text{H}_5 & & \text{C}_2\text{H}_5 \\
| & & | \\
\text{CHOH} & \longleftarrow & \text{HOCH} \\
| & & | \\
\text{C}_6\text{H}_5 & & \text{C}_6\text{H}_{11} \\
\text{Levo.} & & \text{Levo.}
\end{array}
$$
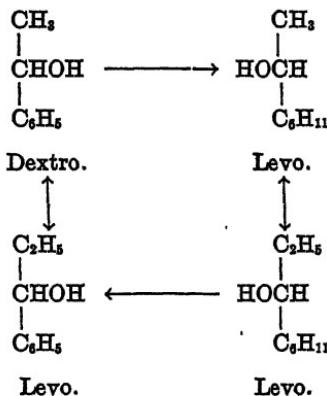
[1] Levene, P. A., and Stevens, P. G., J. Biol. Chem., 87, 375 (1930).

Referring again to the changes in the values of the molecular rotation resulting from hydrogenation, we wish to recall that ethylhexahydrobenzyl carbinol had a considerably higher optical activity than one might have expected. The same is now found to be the case with methylhexahydrobenzyl carbinol.

The ratios of the values of the molecular rotations of the carbinols and their respective hydrogenation products are about the same.

$$\frac{[M]_D^t \text{ ethylbenzyl carbinol}}{[M]_D^t \text{ ethylhexahydrobenzyl carbinol}} = \frac{51.7°}{42.1°} = 1.23$$

$$\frac{[M]_D^t \text{ methylbenzyl carbinol}}{[M]_D^t \text{ methylhexahydrobenzyl carbinol}} = \frac{38.5°}{28.4°} = 1.36$$

Hence, one may assume that the high values found for these hexahydrobenzyl carbinols are not accidental ones.

It was reported in our last paper that in the case of carbinols where the phenyl group was directly attached to the asymmetric carbon atom as much as two-thirds of the material was reduced to the corresponding hydrocarbon. Where the phenyl group was in position (2) from the asymmetric carbon atom, only a small proportion of the material was reduced and where the phenyl group was in position (3), no hydrocarbon was formed. This type of reduction has been the subject of further investigation, the results of which show that not only secondary phenylated carbinols but primary and tertiary as well undergo reduction to hydrocarbons. Aliphatic tertiary carbinols are not reduced, however. Table I shows clearly the facts.

The catalyst employed in all cases was Adams' platinum oxide, the solvent being glacial acetic acid and the pressure of hydrogen 20 to 30 pounds. Two simultaneous reactions take place, hydrogenation of the benzene ring and reduction of the hydroxyl group. When the hydrogenation of the phenyl group is accomplished prior to the reduction of the hydroxyl, the latter then cannot be brought about by further treatment. Thus, the ease of reduction of the phenyl group is a factor in determining the relative amounts of hydrocarbon formed.

Incidentally, it was observed that in the case of triphenyl carbinol, the hydrogenation stops after one phenyl group has been

TABLE I.

Carbinol.	Main product.	Secondary product.
$C_6H_5CH_2OH$	$C_6H_{11}CH_3$	
$C_6H_5CHOHCH_3$	$C_6H_{11}CH_2CH_3$, 60 per cent*	
$C_6H_5CHOHCH_2CH_3$	$C_6H_{11}CH_2CH_2CH_3$, 60 – 90 per cent	
$C_6H_5CHOHCH(CH_3)_2$	$C_6H_{11}CH_2CH(CH_3)_2$ 60 per cent†	
$C_6H_5CHOHC_6H_5$	$C_6H_{11}CHOHC_6H_{11}$ 60 " "	$C_6H_{11}CH_2C_6H_{11}$ 40 per cent
$C_6H_5COH(C_6H_5)_2$	$C_6H_{11}CH_2(C_6H_5)_2 ± 90$ " "	
$C_6H_5CH_2CHOHCH_3$	$C_6H_{11}CH_2CHOHCH_3$	$C_6H_{11}CH_2CH_2CH_3$, trace†
$C_6H_5CH_2CHOHCH_2CH_3$	$C_6H_{11}CH_2CHOHCH_2CH_3$	$C_6H_{11}CH_2CH_2CH_2CH_3$, 8 per cent†
$C_6H_5CH_2CH_2CHOHCH_2CH_3$	$C_6H_{11}CH_2CH_2CHOHCH_2CH_3$, 100 per cent	
$(CH_3)_2COHC_4H_9(n)$	No reduction.	
$(CH_3)_2COHCH(OCH_3)CH_3$	"	

* These values are approximate only.
† Not isolated.

hydrogenated while in the case of diphenyl carbinol (benzhydrol), both phenyl groups are hydrogenated.

In all cases where no reduction took place, as in the case of aliphatic tertiary carbinols, the activity of the catalyst was demonstrated by its capacity to catalyze the hydrogenation of a small amount of ethylallyl carbinol added to the same solution.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Dextro-Methylcyclohexyl Carbinol.*</div>

Reduction of Levo-Methylphenyl Carbinol.[2]—9.0 gm. of the carbinol ($\alpha_D^{26} = -40.20°$; $[\alpha]_D^{26} = -39.8°$; $[M]_D^{26} = -48.8°$[3]) were dissolved in 90 cc. of glacial acetic acid, and shaken with 0.5 gm. of Adams' platinum oxide catalyst with hydrogen at 20 to 30 pounds pressure. The reduction was very rapid. The solution was shaken until no more hydrogen was absorbed and then for several hours longer. The absorption amounted to about 3.6 equivalents of hydrogen. The carbinol was recovered in the usual way. After being dried over potassium carbonate, the material was fractionated at 360 mm. The main part distilled over at 100–106° (bath 135–140°). This consisted largely of the hydrocarbon reduction product. The residual liquid was then distilled at 11 mm. The fraction which boiled at 88° (bath 122°) had the following composition.

4.940 mg. substance: 13.570 mg. CO_2 and 5.645 mg. H_2O.
$C_8H_{16}O$.   Calculated.   C 75.0, H 12.5.
        Found.   " 74.9, " 12.8.

In the homogeneous state the carbinol was dextrorotatory.

$\alpha_D^{30} = +4.12°$; D_4^{30} (estimated) $= 0.865$; $[\alpha]_D^{30} = +4.7°$; $[M]_D^{30} = +6.1°$; $[M]_D^{30} = +6.4°$.[4]

The hydrocarbon distillate was distilled from potassium. It then boiled at 128–130°, which identified it as ethylcyclohexane.[5]

[2] This material was prepared by Levene and Mikeska (Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **70**, 355 (1926)).

[3] Pickard and Kenyon found as a maximum $[M]_D^{26} = -50.9°$ (Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **105**, 1115 (1914)).

[4] Calculated from maximum values of Pickard and Kenyon.

[5] Sabatier and Senderens reported 130° (corrected) (Sabatier, P., and Senderens, J. B., *Compt. rend. Acad.*, **132**, 1255 (1901)).

It had the following composition.

4.770 mg. substance: 14.955 mg. CO_2 and 6.150 mg. H_2O.
C_8H_{16}.   Calculated.   C 85.7, H 14.3.
Found.   " 85.5, " 14.4.

Methylbenzyl Carbinol.

Resolution of Methylbenzyl Carbinol.[6]—The carbinol was resolved through the phthalate by means of brucine.   The maximum activity appeared to have been reached when the phthalate had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 8.08° \times 100}{1 \times 19.47} = + 41.5° \text{ (in absolute alcohol)}.$$

After saponification of the phthalate, the carbinol boiled at 105.5–107° at 15 mm. (bath 135°) and had the following activity in the homogeneous state.

$$\alpha_D^{30} = + 27.77°; D_4^{30} = 0.982; [\alpha]_D^{30} = + 28.3°; [M]_D^{30} = + 38.5°.$$

Calculated from the optical rotation and density curves of Pickard and Kenyon[7] $[M]_D^{20} = +37.4°$.   Pickard's maximum was $[M]_D^{20} = +36.1°$.

Dextro-Methylhexahydrobenzyl Carbinol.

Reduction of Dextro-Methylbenzyl Carbinol.—10 gm. of the carbinol ($\alpha_D^{30} = +27.77°$) were reduced in the usual way with Adams' platinum oxide catalyst.   The total absorption was about 3 equivalents of hydrogen.   The carbinol was isolated as usual.   There was only a small amount of a substance boiling lower than the carbinol.   This was probably the corresponding hydrocarbon. The pure reduced carbinol boiled at 105–106° at 18 mm. (bath 125°) and had the following composition.

4.980 mg. substance: 13.955 mg. CO_2 and 5.775 mg. H_2O.
$C_9H_{18}O$.   Calculated.   C 76.1, H 12.7.
Found.   " 76.4, " 13.0.

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[6] We are indebted to Mr. R. E. Marker for the preparation of this carbinol.

[7] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **105**, 1115 (1914).

In the homogeneous state the reduced carbinol was dextro-rotatory.

$$\alpha_D^{30} = +18.0°; \ D_4^{30} = 0.899; \ [\alpha]_D^{30} = +20.0°; \ [M]_D^{30} = +28.4°.$$

Methylcyclohexane.

Reduction of Benzyl Alcohol.—18.8 gm. of benzyl alcohol (b.p. 200.5–202°) were reduced in the same way as the other carbinols. Almost 4 equivalents of hydrogen were absorbed. The material was isolated in the usual way. Although the ether was carefully fractionated off, a great deal of material was lost, for only 7.5 gm. were finally obtained. On distillation, the main fraction boiled at 100–105°. Redistilled from potassium, it then boiled at 100–101° which identified it as methylcyclohexane.[8] The analysis and refractive index of this sample indicated, however, the presence of a small amount of toluene.

2.335 mg. substance: 7.395 mg. CO_2 and 2.800 mg. H_2O.
C_7H_{14}. Calculated. C 85.7, H 14.3.
            Found.   " 86.4, " 13.4.

$$n_D^{13.5} = 1.4330.$$

The material boiling above 105°, amounting to only about 2 gm., was not further investigated.

Dicyclohexylmethane.

Reduction of Diphenyl Carbinol (Benzhydrol).—10 gm. of the carbinol (m.p. 65.5–66.5°) were reduced in the usual way with Adams' PtO_2 catalyst and the material isolated in the same way as before. On distilling it was apparent at once that two substances were at hand. Several fractions at 0.4 mm. were taken.

. 1st fraction (bath 120°) up to 91°, not viscous.
   2nd   "   ("   125°) 91–101°, last part viscous.
   3rd   "   ("   150–155°) 120–125°, very viscous.

The third fraction crystallized spontaneously to beautiful long heavy plates melting at 63–65°. This identified the substance as dicyclohexyl carbinol.[9] It gave no color reaction with concen-

[8] Sabatier and Senderens found 100.1° (corrected) and $n_D^{13.5} = 1.41705$ (Sabatier, P., and Senderens, J. B., *Compt. rend. Acad.*, **132**, 1255 (1901)).

[9] Sabatier and Mailhe reported 63° (Sabatier, P., and Mailhe, A., *Compt. rend. Acad.*, **139**, 345 (1904)).

trated sulfuric acid, and possessed a pleasant flower-like odor. The total amount of carbinol obtained was about 5.7 gm.

The 1st and 2nd fractions were combined and distilled. The fraction boiling at 85–89° at 0.4 mm. (bath 110°) had the following composition.

2.740 mg. substance: 8.705 mg. CO_2 and 3.305 mg. H_2O.
$C_{13}H_{24}$. Calculated. C 86.7, H 13.3.
Found.   " 86.6, " 13.5.

It was undoubtedly dicyclohexylmethane. The refractive index was $n_D^{19.7} = 1.4785$.[10] Of this hydrocarbon, about 3.5 gm. were isolated.

Diphenylcyclohexylmethane.

Reduction of Triphenyl Carbinol.—10 gm. of triphenyl carbinol (m.p. 159–160°) were suspended in 100 cc. of glacial acetic acid and reduced with Adams' PtO_2 catalyst in the regular way. At first the absorption was rapid but it fell off slowly and ceased after 23 hours. By that time about 4 equivalents of hydrogen had been absorbed. The material was isolated as usual and dried at 100° at 1.0 mm. for 1 hour. An analysis of this liquid showed that the reduction of the carbinol to a hydrocarbon had been practically complete.

2.800 mg. substance: 9.180 mg. CO_2 and 2.410 mg. H_2O.
Found. C 89.4, H 9.7, total 99.1 per cent.

The hydrocarbon was then distilled. The main fraction, 6.2 gm. boiled at 154–156° at 0.5 mm. (bath 185°). This viscous liquid was redistilled at 0.25 mm. yielding a fraction boiling at 156–158° (bath 190°). This was a colorless substance with the following composition.

2.590 mg. substance: 8.630 mg. CO_2 and 2.030 mg. H_2O.
$C_{19}H_{22}$. Calculated. C 91.2, H 8.8.
Found.   " 90.9, " 8.8.

The refractive index was $n_D^{20} = 1.575$.[11]

[10] Eijkman found $n_D^{19.7} = 1.47475$ (Eijkman, T. F., *Chem. Weekbl.*, **3**, 653 (1906) or following papers; abstracted in *Chem. Zentr.*, pt. 2, 1209 (1907)).

[11] Bodroux found $n_D^{20} = 1.571$ (Bodroux, D., *Ann. chim.*, series 10, **11**, 30 (1929)).

HEXOSEMONOPHOSPHATES.

GLUCOSE-3-PHOSPHATE, GLUCOSE-6-PHOSPHATE AND THEIR BEARING ON THE STRUCTURE OF ROBISON'S ESTER.

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In a previous communication it was shown that when the Robison monophosphate was submitted to the action at room temperature of methyl alcoholic hydrogen chloride, the resulting product had the property of a γ-glucoside.[1]   It was concluded from this that the formation of the normal glucoside had been prevented by the presence of the phosphoric radical and that this substituent was consequently on carbon atom (5).   To this ester the structure of glucose-5-phosphate was therefore ascribed.   Position 6 was excluded at that time as the phosphosazone described by Robison[2] was not identical with that prepared from the Harden-Young diphosphate in which the phosphoric radical had been assigned[3,4] to position 6.   Moreover, position 3 had been rendered improbable by the fact that the rate of fermentation of the Robison ester is greater[5] than that of the synthetic product prepared from diacetoneglucose in which only position 3 was open to phosphorylation.

King and Morgan[6] then found that the Robison ester gives, in addition to γ-glucoside at room temperature, a stable form at a higher temperature, and they thus decided that the Robison ester was not glucose-5-phosphate.   Later[7] they came to the conclusion that it was glucose-3-phosphate.

[1] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **81**, 279 (1929).

[2] Robison, R., *Biochem. J.*, **16**, 809 (1922).

[3] Morgan, W. T. J., and Robison, R., *Biochem. J.*, **22**, 1270 (1928).

[4] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **80**, 633 (1928).

[5] Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 621 (1928).

[6] King, E. J., and Morgan, W. T. J., *J. Soc. Chem. Ind.*, **48**, 143 (1929).

[7] King, E. J., and Morgan, W. T. J., *J. Soc. Chem. Ind.*, **48**, 296 (1929).

On the other hand, we have found[8] that the synthetic ester prepared through diacetoneglucose forms an anhydrosazone containing no phosphorus, which further distinguishes this ester from Robison's.

In view of the fact that these two esters are different, the final conclusion of King and Morgan can be correct only if the synthetic ester is not glucose-3-phosphate as we had assumed. For this reason we were led to reexamine this ester in order to establish its structure more conclusively, and in this paper we are presenting our new data. They confirm our belief that the synthetic ester is glucose-3-phosphate. Also, inasmuch as one of the structures which entered into consideration was glucose-6-phosphate, this substance was also prepared and is described herein.

We may first discuss the structure of the synthetic ester from diacetoneglucose on the basis of all data now available. We find that under the conditions used in the phosphorylation, there is no appreciable hydrolysis of the acetone groups. Thus unless there is a migration of one of them during the phosphorylation, the phosphoric acid group in the diacetonephosphate is located in position 3.[9] Hence, in the hexosemonophosphate obtained after removing the acetone groups, the phosphoric acid group would be in position 3 unless it had migrated during the process of hydrolysis.[10]

The migration of either the acetone groups or the phosphoric acid group is excluded by the properties of the final hexosemonophosphate; it forms a stable and an unstable glucoside (Fig. 1), the phosphoaldonic acid prepared from it gives two lactones whose

[8] Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **83**, 619 (1929).

[9] Karrer, P., and Hurwitz, O., *Helv. Chim. Acta*, **4**, 728 (1921). Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **54**, 805 (1922); **57**, 317 (1923); **60**, 173 (1924). Freudenberg, K., and Brauns, F., *Ber. chem. Ges.*, **55**, 3233 (1922). Freudenberg, K., and Doser, A., *Ber. chem. Ges.*, **56**, 1243 (1923). Ohle, H., *Ber. chem. Ges.*, **57**, 403 (1924). Anderson, C. G., Charlton, W., and Haworth, W. N., *J. Chem. Soc.*, 1329 (1929).

[10] While this work was being prepared for press, an article by Josephson and Proffe appeared (*Ann. Chem.*, **481**, 91 (1930)) in which the authors showed that in the phosphate obtained through diacetoneglucose the phosphoric acid group did not change position under the influence of either acids or alkalies. Regarding this synthetic hexosemonophosphate to be glucose-3-phosphate, they disagreed with the conclusion of King and Morgan that the Robison ester is glucose-3-phosphate.

rates of formation are roughly the same as those of ordinary $<1,4>$ and $<1,5>$ lactones (Fig. 2), and, as already mentioned, the ester gives a 3-6-anhydrohexosazone instead of a phosphohexosazone as does the Harden-Young ester.[11]

Thus, by arguments similar to those used in the discussion of the Robison ester,[7] positions 4, 5, and 6 may be excluded and only 1 and 2 remain to be considered. These positions could not be reconciled with the formation of the 3-6-anhydrohexosazone. Thus the phosphoric group in this ester appears to be definitely in position 3.

On the other hand, as stated above, the synthetic ester and the Robison ester are distinctly different; they have different rates of fermentation, particularly in the presence of arsenate[5] (Figs. 3 and 4), have different rotations both as salt[12,13] and as free acid,[12] and differ in their osazone formation.[2,8] Although the Robison ester has apparently never been prepared in an entirely pure form, still these differences are sufficiently pronounced to make it certain that the Robison monophosphate cannot be identical with the synthetic ester from diacetoneglucose.

These facts can be reconciled with the view of King and Morgan that the Robison ester is glucose-3-phosphate, only on the assumption of a difference in the configuration of the hexoses of the two esters. However, we now find that the synthetic and Robison esters, on dephosphorylation by intestinal phosphatase under identical conditions, both yield glucose, isolated as glucosazone. If, then, the two esters are not derivatives of the same hexose it means that a Walden inversion has been produced in only one ester in the process of enzymic dephosphorylation. This possibility does not appear very probable.

If, however, we admit that the Robison ester is not glucose-3-phosphate, and take into consideration the findings of King and Morgan which apparently exclude position 5, then we must again consider glucose-6-phosphate as a possible structure. For this reason the preparation of this ester was desirable. Inasmuch as

[11] von Lebedev, A., *Biochem. Z.*, **28**, 213 (1910).

[12] Komatsu, S., and Nodzu, R., *Mem. Coll. Sc., Kyoto Imp. Univ.*, **7**, 377 (1924). Nodzu, R., *J. Biochem.*, **6**, 31 (1926).

[13] Josephson, K., and Proffe, S., *Ann. Chem.*, **481**, 91 (1930).

Ohle had found that monoacetoneglucose could be sulfonated,[14] benzoylated,[15] or toluenesulfonated[16] in position 6 we were led to try the phosphorylation under similar conditions. The resultant ester, isolated and purified as the brucine salt, gave a phosphosazone melting at 151–152° and apparently identical with those obtained from the Harden-Young and Neuberg esters as would be expected if all three were substituted in position 6. In addition, this new ester is of great interest for, alone among the synthetic hexosephosphates, it has a high rate of fermentation, one practically identical with that of the Robison ester (Figs. 3 and 4). Likewise the brucine and barium salts are quite similar in their properties to those of the Robison. These facts suggest that the Robison ester might be glucose-6-phosphate but we are then confronted with the differences reported for the melting points of the osazones of the Robison and Harden-Young esters, that of the former having been given as 139° and the latter 151°. Is it not possible that the first of these two has not been prepared in an entirely pure state? Had we been in possession of a sufficiently pure sample of the Robison ester from which to prepare the osazone, then a comparison might have given a conclusive answer. We may mention, however, that a sample of the Robison ester that had been twice crystallized as the brucine salt but which according to Willstätter's method contained only 60 per cent of the aldose derivative, gave a 19 per cent yield of purified osazone, identical with that from the Harden-Young diphosphate. Further purification of the Robison ester is now in progress in order to permit the comparison of its osazone and other derivatives with those of our synthetic 6-phosphate.

Should the Robison ester prove not to be glucose-6-phosphate then carbon atoms 4 and 5 will again come to the front as possible positions of the phosphoric acid group. Renewed investigation will be required to determine whether, of the two glucosides of the Robison ester, one actually has the <1,4> and the other the <1,5> lactal structure.

[14] Ohle, H., *Biochem. Z.*, **136**, 428 (1923).

[15] Ohle, H., *Ber. chem. Ges.*, **57**, 403 (1924).

[16] Ohle, H., and Dickhauser, E., *Ber. chem. Ges.*, **58**, 2593 (1925).

I. Analytical.

A. Aldose Determination by Willstätter Method.—For the hypoiodate titrations the following procedure was employed throughout.

The sample, containing not over 0.35 mm of aldose, was placed in a flask and diluted to a volume of 20 cc. (In the case of acid samples, a quantity of 0.2 N NaOH equivalent to the acid was added before diluting.) There were then added 10.0 cc. of 0.1 N iodine solution and, over a period of $1\frac{1}{2}$ to 2 minutes, 10 cc. of 0.2 N NaOH. The mixture was left at room temperature (about 20°) for 15 minutes, acidified, and titrated with 0.1 N thiosulfate solution.

Determinations made in this manner were consistently low but were entirely reproducible. A preparation of barium hexosemonophosphate which had been purified by three precipitations with alcohol contained, according to the titration, 90.6 per cent aldose. After further purifying this product by recrystallizing twice as the brucine salt, the value rose only to 91.2 per cent. That these low figures may be due to the method used and not to impurity of the ester is suggested by the fact that still lower values (as low as 60 per cent) were obtained if the concentrations were increased and the alkali was added instantly.

B. Acetone Determination.—The sample in which the acetone was to be determined was placed in a test-tube fitted with a ground glass connection and the tube was heated in a boiling water bath for 45 minutes. During this time a stream of air was bubbled through the mixture in the tube, and carried the acetone to an absorber where it was converted to iodoform by an alkaline hypoio-

date solution. The amount of acetone was calculated from the quantity of hypoiodate which was used up.

In the case of the samples from the phosphorylation mixture, it was necessary to prevent hydrolysis of the acetone during the determination, so the mixture was made alkaline with sodium hydroxide. 1 cc. of the pyridine solution and 2 cc. of 5 N NaOH were diluted to 20 cc. and this mixture was heated and aerated as described. For control, a known 2.0 mg. of acetone were added to such an alkaline pyridine mixture and after the 45 minutes heating 1.94 mg. were found on titration. This is a 97 per cent recovery which is well within the limits of experimental error.

II. Hexosemonophosphate from Diacetoneglucose.

A. Preparation.—In attempting to prepare pure diacetone-glucose phosphate by the method previously described,[8] it was found very difficult to obtain a product which had not suffered more or less hydrolysis to the monoacetonephosphate. Acetone analyses were performed and it was found that the hydrolysis occurred during the isolation of the product and not during the phosphorylation, the diacetone remaining intact during this opera-tion. The procedure was therefore modified by omitting the isola-tion of the diacetonephosphate, the product being directly hydro-lyzed and the monophosphate isolated as the brucine salt. The preparation was as follows:

20 gm. of recrystallized diacetoneglucose were dissolved in 100 cc. of dry pyridine and cooled to −40° to −50°. To this, there was added a solution of 6.5 cc. of redistilled phosphorus oxychlo-ride in 25 cc. of cold (−30° to −35°) dry pyridine. The mixture was removed from the cooling mixture, stirred for a few minutes, and placed in an ice-salt bath for 2 hours. It was then recooled to −35° and 15 cc. of a 10 per cent solution of water in pyridine were added in small portions, cooling between each addition and keeping the temperature below −20°. After the addition of the aqueous pyridine, ice was added and then ice water.

Acetone determinations were performed to ascertain whether an appreciable hydrolysis of the diacetoneglucose had occurred during the phosphorylation. 1 cc. of the pyridine solution of the diacetoneglucose, before phosphorylation, was analyzed as de-scribed above, and 0.31 mg. of acetone was found. Since there were 200 mg. of diacetoneglucose present, this corresponds to a

0.35 per cent hydrolysis of both groups or to 0.7 per cent hydrolysis to monoacetoneglucose. An equivalent quantity of the solution analyzed after phosphorylation gave only 0.21 mg. of acetone which is a smaller rather than a larger amount. Thus it is evident that no cleavage of the acetone groups. preceded the phosphorylation.

Four batches of material, equivalent to 80 gm. of diacetone-glucose, were combined and concentrated under reduced pressure. Water was added from time to time and the distillation was continued until little pyridine remained. 200 cc. of 5 N hydrochloric acid were added, and the mixture was diluted to 1 liter and kept at 38-40° for 60 hours. It was cooled, and the hydrochloric acid was removed by adding a suspension, in water, of freshly precipitated, washed, silver carbonate. At the end-point, when the hydrochloric acid was just removed, the mixture was made acid with 5 N sulfuric acid, and an additional 10 cc. per liter of this acid were then added. The acid mixture was stirred for several minutes and then suction-filtered. The precipitate was suspended in 1½ liters of 0.05 N sulfuric acid, stirred well, and refiltered. The solutions were combined, treated with warm saturated barium hydroxide solution until the sulfate was exactly removed, and filtered.

10.0 cc. of this final solution required 6.8 cc. of 0.1 N sodium hydroxide for neutralization (phenolphthalein). The total volume being 4.7 liters this is a yield (calculated from the phosphorus oxychloride) of 55 per cent of the theoretical. A solution of brucine in methyl alcohol was added to a pH of 7.5 to 7.7, the mixture was filtered with charcoal, and concentrated under reduced pressure. A large amount of almost colorless, crystalline material separated. This was filtered off and the concentration was resumed. The crystalline product was recrystallized from methyl alcohol and then from water. It was washed with methyl alcohol and with acetone and was then dried. The yield was 126 gm. or 41 per cent. From the various mother liquors an additional 29 gm. (9 per cent) were obtained.

The product was a dibrucine salt of a hexosemonophosphate as shown by the analysis:

11.985 mg. substance: 0.574 cc. N (756 mm. and 30°).
5.490 "   "   : 0.278 " " (754 "   " 37°).
5.405 "   "   : 11.430 mg. ammonium phosphomolybdate (Pregl).
         $C_{52}H_{65}O_{17}N_4P$. Calculated. N 5.34, P 2.96.
                         Found.   " 5.32, " 3.07.
                                     " 5.65.

The rotation was

$$[\alpha]_D^{25} = \frac{-\ 1.69° \times 100}{2 \times 2.0} = -\ 42.3° \text{ (in pyridine)}.$$

Starting from a different lot of diacetoneglucose, the phosphorylation and hydrolysis were performed in the same manner but the ester was isolated by repeated precipitation of the barium salt from water with an equal volume of 95 per cent alcohol. The barium salt was converted to the brucine salt and this was twice crystallized from 50 per cent acetone and then once from 95 per cent methyl alcohol. The analysis was similar to that of the substance above and the rotation was

$$[\alpha]_D^{25} = \frac{-\ 1.67° \times 100}{2 \times 2.0} = -\ 41.8° \text{ (in pyridine)}.$$

The substance was rather insoluble in water, ethyl alcohol, or 95 per cent methyl alcohol, but was quite soluble, hot, in all of these. It was not very soluble in absolute methyl alcohol even on heating and was quite insoluble in acetone. It was easily crystallized from 95 per cent methyl alcohol, water, or 50 per cent acetone.

A comparison of different fractions gave no indication of appreciable amounts of any isomeric substance in the purified product.

80 gm. of the recrystallized brucine salt were dissolved in 300 cc. of warm 70 per cent methyl alcohol. The solution was cooled and a saturated solution of barium hydroxide in methyl alcohol was added slowly and with stirring until the mixture was alkaline to phenolphthalein. An equal volume of acetone was added, the mixture was stirred for a few minutes and then suction-filtered. The barium salt was taken up in water, concentrated under reduced pressure to about 300 cc., and filtered to remove the brucine which had separated. The filtrate was concentrated to a volume of 150 cc., filtered with charcoal, and precipitated with an equal volume of 95 per cent alcohol. The precipitate was washed with 50 per cent alcohol and redissolved in water. The solution was again concentrated under reduced pressure and was reprecipitated with an equal volume of 95 per cent alcohol. The precipitate was washed with 50 per cent, 95 per cent, and absolute alcohol and air-dried.

It was then powdered and dried to constant weight over phosphorus pentoxide in a vacuum desiccator.

The analysis corresponded to a barium hexosemonophosphate.

4.095 mg. substance: 22.745 mg. ammonium phosphomolybdate (Pregl).
0.0918 gm.   "   : 0.0528 gm. BaSO₄.

$C_6H_{11}O_9PBa$.   Calculated.   P 7.84, Ba 34.74.
                  Found.   " 8.06, " 33.84.

The rotation was

$$[\alpha]_D^{27} = \frac{+ 0.53° \times 100}{2 \times 1.0} = + 26.5°.$$
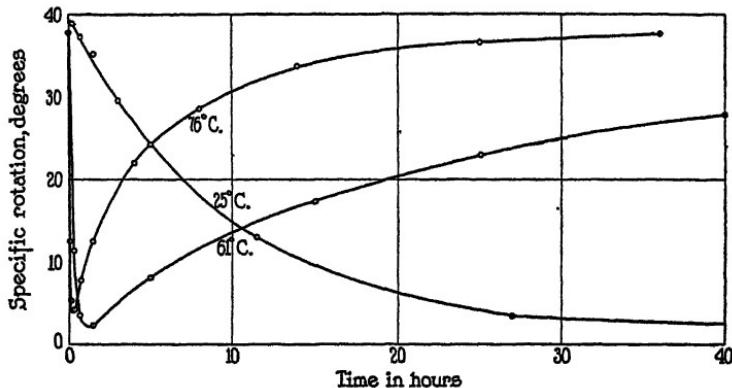
FIG. 1. Specific rotations during glucoside formation.

The rotation of the free acid (the barium having been quantitatively removed with sulfuric acid) was

$$[\alpha]_D^{27} = \frac{+ 0.49° \times 100}{2 \times 0.62} = + 39.5°.$$

B. Glucoside Formation.   Experiment I.—1.2 gm. of the pure barium salt were dissolved in 45 cc. of dry methyl alcohol containing 0.45 gm. of HCl gas (0.5 per cent free) and kept at 25.0° in a thermostat.   The rotations, measured in a 4 dm. tube at 25° with sodium (D) light, are given in Table I, and the specific rotations calculated from these data, are given in Fig. 1.

Experiment II.—A mixture similar to the above was prepared, and 6 cc. portions were placed in tubes which were sealed, the oper-

ations all being conducted at about 5°. The sealed tubes were
placed in a bath of boiling chloroform (about 61°) for varying
periods, and on removal were recooled in an ice bath. They were
opened, shaken well to suspend the barium chloride which had
precipitated, and 5.0 cc. of solution were removed and placed in a
test-tube. This was cooled in ice, and 1.0 cc. of water was added to
dissolve the barium chloride. After shaking, the rotation was
read in a 2 dm. tube at 25° using sodium (D) light. Readings
made immediately, and again after 5 to 10 minutes were identical
within the limits of error of the polariscope, which shows that no

TABLE I.

Observed Rotations during Glucoside Formation at 25°.

Time.		Rotation.	Time.		Rotation.	Time.	Rotation.
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*degrees*
0	15	4.16	5	00	2.59	71	0.17
0	45	3.99	11	30	1.40	117	0.27
1	30	3.76	27		0.37	189	0.44
3	05	3.16	46		0.23		

TABLE II.

Observed Rotations during Glucoside Formation at 61°.

Time of heating.		Rotation.	Time of heating.		Rotation.	Time of heating.	Rotation.
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*degrees*
0	00	1.68	1	30	0.06	25	1.02
0	20	0.51	5	00	0.36	40	1.24
0	40	0.16	15	00	0.78		

significant hydrolysis of the glucosides was taking place under the
conditions used. The readings are given in Table II, and the
specific rotations calculated from these data are plotted in Fig. 1.

Experiment III.—Experiment II was repeated with the excep-
tion that the tubes were heated in a boiling carbon tetrachloride
bath (76°). The readings are given in Table III and the specific
rotations calculated from these data are plotted in Fig. 1.

Experiment IV.—6.0 gm. of dry barium monophosphate (not
prepared through the brucine salt, but having the same specific
rotation as the sample which had been thus purified) were dissolved

in 225 cc. of dry methyl alcohol containing 1.8 gm. of hydrogen chloride gas, and cooled in ice water.   Portions of the clear solution were transferred to round bottom flasks which were sealed and heated in a boiling carbon tetrachloride bath for 25 minutes in one case, and for 14 hours in the other.   The flasks were opened and after shaking to mix in the precipitate, a definite volume of solution was removed and neutralized with an alcoholic solution of barium hydroxide.   3 volumes of ether were added and the

TABLE III.

Observed Rotations during Glucoside Formation at 76°.

Time of heating.		Rotation.	Time of heating.		Rotation.	Time of heating.	Rotation.
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*degrees*
0	00	1.68	0	40	0.35	14	1.50
0	05	0.56	1	30	0.56	25	1.63
0	10	0.24	4	00	0.98	36	1.67
0	20	0.19	8	00	1.27		

TABLE IV.

Glucoside Formation at 76°.

Time of heating.	Gm. from 90 cc. of solution.	0.1 N thiosulfate.		Calculated aldose.		Percentage.		
		Original.	After hydrolysis.	Original.	After hydrolysis.	Free aldose.	γ-Glucoside.	Normal glucoside (by difference).
		cc. per gm.	*cc. per gm.*	*gm.*	*gm.*			
None.	2.40	41.80		2.19		(100)		
25 min.	4.80	2.14	16.80	0.23	1.76	10	70	20
14 hrs.	3.77	1.36	4.32	0.11	0.35	5	10	85

mixture was centrifuged.   The precipitate was suspended in more ether and centrifuged, and this was repeated once more.   The barium salt was dried in a vacuum desiccator over calcium chloride and then over phosphorus pentoxide to constant weight.   A colorimetric phosphate determination proved that no appreciable hydrolysis of the phosphoric group had occurred.

   The dried crude salts were weighed, and an aliquot portion was dissolved in water and used for hypoiodate titration, before and

after hydrolysis. For the hydrolysis, the solution was mixed with an equal volume of 0.30 N hydrochloric acid (so that the solution was 0.1 N in free hydrochloric acid) and heated in a boiling water bath for 10 minutes. It was then cooled, neutralized with sodium hydroxide, and diluted to a definite volume. Under these conditions, known γ-glucosides are almost completely hydrolyzed whereas normal glucosides remain nearly intact. In making the calculations, the value found for the original ester is taken as 100
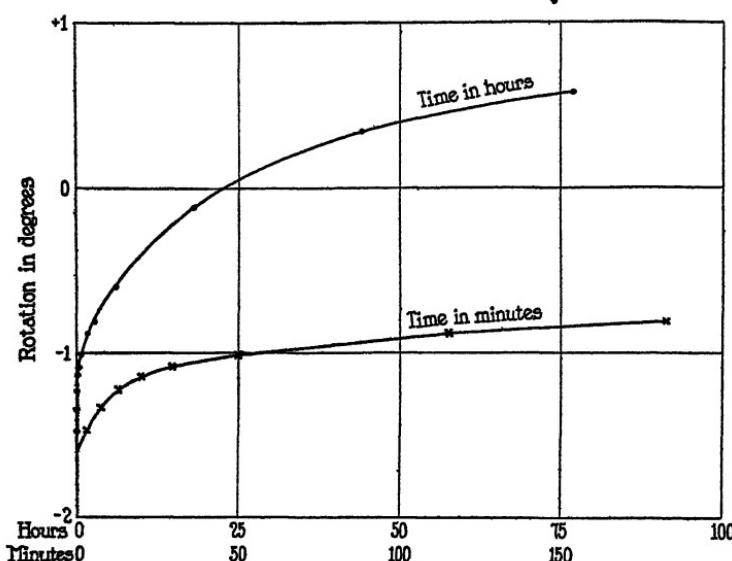
Fig. 2. Observed rotations during lactone formation. Two different time scales are used to show the break in the curve after about an hour.

per cent and the rest of the values are calculated from this. The percentage of normal glucoside is obtained by subtracting γ-glucoside and free aldose from 100. The results, calculated in this way, are given in Table IV.

The initial reaction product thus appears to be an unstable glucoside, and the latter one a stable form.

C. *Aldonic Acid and Lactone Formation.*—12 gm. of the pure barium monophosphate were dissolved in 60 cc. of water and added to a solution of 7.8 gm. of iodine and 15 gm. of barium iodide in

30 cc. of water. Over a period of 10 minutes, 205 cc. of 0.59 N barium hydroxide solution were added with stirring. The mixture was allowed to stand at room temperature for 15 minutes and was then centrifuged. The precipitate was suspended in water and dissolved by the careful addition of hydrochloric acid. Sulfur dioxide gas was passed in until the iodine just disappeared. The mixture was centrifuged and the solution was filtered with charcoal. Barium hydroxide solution was added until the mixture was alkaline to phenolphthalein and it was then centrifuged. The precipitate was suspended in water and recentrifuged and this was repeated once more. The barium salt was then suspended in water, and a little sulfuric acid was added, the mixture was shaken well, and more sulfuric acid was added, this being continued until

TABLE V.

Observed Rotations during Lactone Formation.

Time.		Rotation.	Time.		Rotation.	Time.	Rotation.
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*degrees*
0	03	−1.47	0	50	−1.01	44	+0.34
0	08	−1.34	1	55	−0.88	77	+0.58
0	13	−1.23	3	03	−0.81	142	+0.75
0	30	−1.14	6	05	−0.60		
0	40	−1.09	18	05	−0.12		

the barium was exactly removed. 10.3 cc. of 5 N sulfuric acid were required. The mixture was centrifuged and the clear solution was neutralized with 5 N sodium hydroxide, 10.2 cc. being required (phenolphthalein). The solution was concentrated under reduced pressure to a small volume, absolute alcohol and benzene were added and the distillation was continued to remove all but traces of water. The alcohol and benzene were then removed and the residue was dried in a high vacuum over phosphorus pentoxide. It was then powdered and redried to constant weight. Yield 5.5 gm. The phosphorus content corresponded to a trisodium salt of a phosphohexonic acid. It analyzed as follows:

7.360 mg. substance: 45.420 mg. ammonium phosphomolybdate (Pregl). $C_5H_{10}O_{10}PNa_3$. Calculated. P 9.07. Found. P 8.96.

0.25 gm. of this sodium salt was dissolved in water, 1.46 cc. of 1.0 N hydrochloric acid (calculated for 2 equivalents) were added, and

the mixture was diluted to 5.0 cc. It was filtered into a 2 dm. polariscope tube and the readings were made with sodium (D) light. The tube was kept at room temperature, which was 25–27°. The rotations are given in Table V, and are plotted in Fig. 2, two different time scales being used in order to show better the break in the curve after about an hour.

III. Hexosemonophosphate from Monoacetoneglucose.

20 gm. of recrystallized monoacetoneglucose were dissolved in 100 cc. of dry pyridine and cooled to −35°. A solution of 8.0 cc. of redistilled phosphorus oxychloride in 25 cc. of cold (−30°) dry pyridine was added in six portions, the mixture being recooled to −35° before each addition. The temperature was never above −20° during the phosphorylation. The mixture was kept in an ice-salt mixture for 2 hours and ice and ice water were then added. The solution was concentrated under reduced pressure until nearly all the pyridine was gone, water being added from time to time. It was diluted to 160 cc. and 40 cc. of 5 N hydrochloric acid were added. The mixture was kept at 38–40° for 60 hours and was then cooled. Removal of the chloride, and conversion to the brucine salt were performed exactly as in the case of the phosphate from the diacetoneglucose.

The solution of the brucine salt was filtered with charcoal and concentrated to about 200 cc. 3 volumes of acetone were added and the mixture was cooled and stirred. The brucine salt crystallized with great ease.

After the mother liquors were worked up a total of 42 gm. of the brucine salt was obtained. A portion was recrystallized by dissolving in water, filtering, and adding 3 volumes of acetone. After about an hour, the crystals were filtered off, washed with acetone, and air-dried.

The analysis corresponded to a dibrucine salt of a hexosemonophosphate.

5.405 mg. substance: 0.275 cc. N (753 mm. and 31°).
4.035 "   "   : 8.560 mg. ammonium phosphomolybdate (Pregl).
$C_{52}H_{66}O_{17}N_4P$. Calculated. N 5.34, P 2.96.
                         Found.   " 5.65, " 3.08.

The rotation of the brucine salt was

$$[\alpha]_D^{25} = \frac{-1.38° \times 100}{2 \times 1.4} = -49.3° \text{ (in pyridine).}$$

and

$$[\alpha]_D^{25} = \frac{-0.62° \times 100}{2 \times 2.0} = -15.5° \text{ (in 20 per cent alcohol).}$$

Robison[2] originally found for the brucine salt of his ester $-23.4°$ in water at 6.6 per cent concentration.

The brucine salt was quite soluble in water and methyl alcohol, less in absolute alcohol, and very little in acetone.

A portion of the brucine salt was dissolved in water, made alkaline to phenolphthalein with barium hydroxide solution, stirred a few minutes, and filtered. The filtrate was concentrated under reduced pressure to a small volume and precipitated with an equal volume of alcohol. After being washed with 50 per cent alcohol it was redissolved and reprecipitated. It was again washed with 50 per cent alcohol and then with absolute. Finally it was dried.

The rotation of the barium salt was

$$[\alpha]_D^{25} = \frac{+0.52° \times 100}{2 \times 2.0} = +13.0° \text{ (in water).}$$

and that of the free acid (the barium having been quantitatively removed with sulfuric acid)

$$[\alpha]_D^{25} = \frac{+0.68° \times 100}{2 \times 1.31} = +25.9°.$$

Robison[2] first gave 12.5° and 25.0° for the corresponding values for his ester, but in his most recent communication[17] finds 21.0° for the rotation of the barium salt of his purest preparation. It is probable that neither the brucine nor barium salt given above is quite pure as there was not sufficient material for satisfactory fractionation.

The barium was exactly removed from 2.0 gm. of the barium salt with sulfuric acid, phenylhydrazine and acetic acid were added, and the mixture was heated on the steam bath, the osazone being filtered off from time to time till little more formed. It was col-

[17] Robison, R., and King, E. J., *J. Soc. Chem. Ind.*, **48**, 143 (1929).

lected and dried, and washed first with methyl alcohol and then with chloroform. After being redried, the weight was 0.7 gm. (26 per cent). It melted at 151–152°, as did a sample of osazone from the hexosediphosphate and an intimate mixture of the two.

The rotation in pyridine-absolute alcohol (2:3) was −45.6° after 15 minutes and

$$[\alpha]_D^{25} = \frac{-0.22° \times 100}{0.5 \times 1.4} = -31.4° \text{ after 1 hour (equilibrium).}$$

Neuberg[18] found −50.9° and −36.0° respectively for the osazone from the Harden-Young diphosphate.

The analysis corresponded to a phenylhydrazine salt of a phosphohexosazone.

5.930 mg. substance: 23.100 mg. ammonium phosphomolybdate (Pregl).
4.565 "   "   : 0.621 cc. N (758 mm. and 29°).
$C_{24}H_{31}O_7N_6P$.   Calculated.   N 15.38, P 5.68.
                         Found.   " 15.33, " 5.65.

IV. Robison Monophosphate.

Glucose was fermented with yeast juice, the inorganic phosphate being followed colorimetrically and kept at 0.01 to 0.04 molal by frequent additions of a 3 molal phosphate solution. After 3 to 4 hours the fermentation was interrupted by the addition of trichloroacetic acid, the diphosphate was removed as the barium salt, and the monophosphate was precipitated twice as the lead salt and then converted to the barium salt. This was precipitated from its aqueous solution with alcohol, the barium was removed with sulfuric acid, and the brucine salt was formed. The aqueous solution of the brucine salt was concentrated under reduced pressure and an insoluble portion was filtered off. After addition of 3 volumes of acetone to the filtrate, the brucine phospho-ester crystallized. This was filtered off, redissolved, and crystallized in the same way. It was then converted to the barium salt. A portion of the dried barium salt was used for the hypoiodate titration.

0.120 gm. required 3.8 cc. of 0.1 N thiosulfate, equivalent to 0.076 gm. of barium monophosphate or 63 per cent.

From 3 gm. of this material the barium was exactly removed

[18] Neuberg, C., and Reinfurth, E., *Biochem. Z.*, **146**, 589 (1924).

with sulfuric acid and to a portion of the solution equivalent to 2.6 gm., phenylhydrazine and acetic acid were added. The mixture was heated on the steam bath and removed from time to time to filter off the osazone which had formed. The yield of crude - dry osazone was 1.2 gm. It was washed with methyl alcohol and with chloroform and was then dried. The yield was 0.7 gm. The soluble portion was dark and tarry but apparently more of the same material was contained in it. The melting point of the purified material was 151–152° and the mixed melting point with a sample of Harden-Young osazone was the same, 151–152°. The rotation was $[\alpha]_D^{25} = -57.2°$ after 2 minutes (in pyridine-absolute alcohol, 2:3), and

$$[\alpha]_D^{25} = \frac{-0.23° \times 100}{0.5 \times 1.4} = -32.9° \text{ after 1 hour (equilibrium).}$$

Neuberg[18] found −50.9° after 15 minutes and −36.0° after 1 hour for the osazone from the Harden-Young diphosphate. The analysis was as follows:

3.630 mg. substance: 0.514 cc. N (758 mm. and 30°).
3.790 " " : 13.825 mg. ammonium phosphomolybdate (Pregl).
$C_{24}H_{31}O_7N_6P.$ Calculated. N 15.38, P 5.68.
Found. " 15.90, " 5.29.

V. Enzymic Dephosphorylation of Esters.

Experiment I.—1.5 gm. of Robison barium monophosphate (an earlier preparation having $[\alpha]_D^{25} = +12.4°$) were dissolved in 30 cc. of water and adjusted to pH 8.4. 75 mg. of dry intestinal phosphatase[19] powder were added and the mixture was placed in a thermostat at 40°. Barium hydroxide solution was added from time to time to restore the pH and at 2 hours another 75 mg. and at 4½ hours 30 mg. of enzyme were added. At 5½ hours a determination of the amount of inorganic phosphate indicated about 90 per cent hydrolysis of the ester. The hydrolysis was allowed to proceed for 3 hours more, and the precipitated barium phosphate was then removed by centrifuging. The solution was precipitated · with 3 volumes of absolute alcohol, ·filtered, concentrated to a volume of 15 cc. and precipitated with 10 volumes of absolute

[19] Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **88**, 753 (1930).

alcohol. The precipitate was filtered off using a little Filter-Cel, and the solution was concentrated to a volume of 10 cc.

To this solution, phenylhydrazine and acetic acid were added; the mixture was diluted and heated on the.steam bath. The osazone which formed was filtered off, dried, and extracted with methyl alcohol. Yield 0.25 gm.

The substance contained no phosphorus.

$$[\alpha]_D^{25} = \frac{-0.20° \times 100}{0.5 \times 0.45} = -89° \text{ after 15 minutes (in pyridine-absolute alcohol, 3:2).}$$

$$[\alpha]_D^{25} = \frac{-0.06° \times 100}{0.5 \times 0.45} = -27° \text{ after 24 hours.}$$

The melting point was 206–207° as was the melting point of an intimate mixture with a sample of pure glucosazone.

Experiment II.—This was a duplicate of Experiment I as to proportions and times, except that 2.0 gm. of the barium monophosphate from the diacetoneglucose were used. After $8\frac{1}{2}$ hours the hydrolysis was nearly complete as shown by a phosphate determination. The isolation and conversion to the osazone were similar to those described above. The yield of purified osazone was 0.37 gm. It contained no phosphorus.

$$[\alpha]_D^{25} = \frac{-0.18° \times 100}{0.5 \times 0.48} = -75° \text{ after 15 minutes (in pyridine-absolute alcohol, 3:2).}$$

$$[\alpha]_D^{25} = \frac{-0.05° \times 100}{0.5 \times 0.48} = -21° \text{ after 24 hours.}$$

The melting point was 205–206° and the mixed melting point with pure glucosazone was 206–207°.

Experiment III.—In order to exclude the possibility of the presence of allosazone in the product, Experiment II was repeated on a larger scale. 4.6 gm. (dry) of the pure barium salt (through the brucine salt) were dissolved in 100 cc. of water and the pH was adjusted to 8.7 with barium hydroxide solution. 0.5 gm. of dry enzyme was added and the mixture was placed in a thermostat at 40°. The pH was frequently readjusted to 8.7 by the addition of barium hydroxide solution. After 4 hours the barium phosphate was centrifuged off and an additional 0.5 gm. of enzyme

was added.   After 1½ hours more, the pH shôwed no further tend-
ency to change and as a phosphate determination indicated 94
per cent hydrolysis, the mixture was removed from the thermostat.
After centrifuging off the precipitate, the solution was concentrated
under reduced pressure to 20 cc. volume and precipitated with 10
volumes of absolute alcohol.   After stirring, the precipitate coagu-
lated nicely and was filtered off, a little Filter-Cel being used.   To
the clear filtrate there were added 5 cc. of phenylhydrazine, 5 cc.
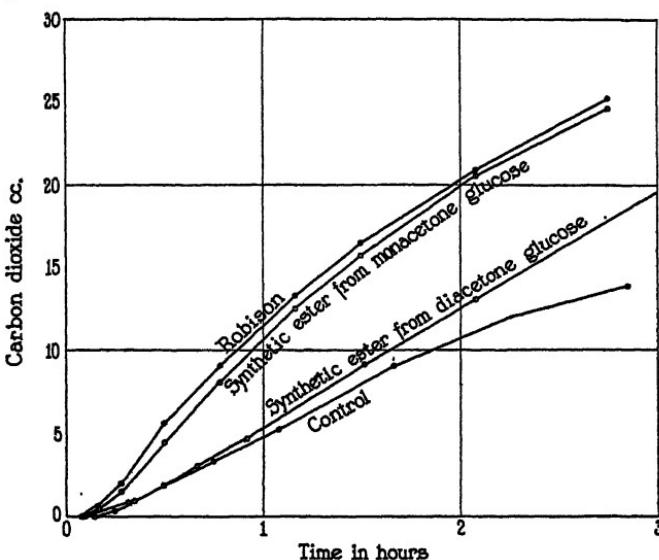
FIG. 3. Rate of fermentation of various esters with "lager" zymin.

of glacial acetic acid, and water to 100 cc.   The mixture was
heated on the steam bath for 1 hour, the osazone was filtered off,
and the mixture reheated.   This was continued until little further
osazone was formed.   The combined material was dried.   It was
extracted overnight with cold methyl alcohol, filtered, and washed
with a little more methyl alcohol.   The alcoholic extracts on con-
centration gave 0.3 gm. of dark tarry material from which only
a little glucosazone (about 0.1 gm.) was obtained.   No allosazone
could be isolated.   The alcohol-washed osazone, which was canary-
yellow in color, after drying weighed 1.6 gm.

. The substance contained no phosphorus and the nitrogen content corresponded to a hexosazone.

5.000 mg. substance: 0.712 cc. N (752 mm. and 31°).
$C_{18}H_{22}O_4N_4$. Calculated. N 15.63. Found. N 15.82.

The melting point was 208–210° and the melting point of an intimate mixture with a known glucosazone was the same, 208–210°.

The yield of 1.6 gm. is 38 per cent based on the original hexosemonophosphate while from 0.9 gm. of glucose there was obtained, under the same conditions, 0.5 gm. of osazone or 28 per cent.
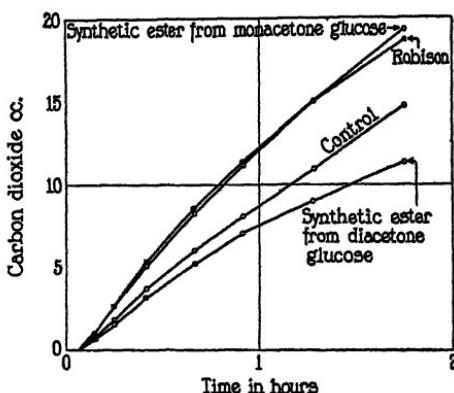
FIG. 4. Rate of fermentation of various esters with "ale" zymin.

VI. Enzymic Fermentations of Esters.

The fermentations were done using the methods and apparatus previously described.[20] For Robison ester a sample from an earlier preparation was used, the barium salt having an $[\alpha]_D^{25} = +12.4°$. The synthetic esters employed were those described above. All esters were used in the form of their sodium salts. As differences between the Robison and synthetic esters were previously found to be emphasized in the presence of arsenate, this was used throughout.

"Lager" Zymin.—The esters were 0.06 molal, arsenate 0.004 molal, and hexosediphosphate 0.005 molal throughout. In Fig. 3 it will be observed that the synthetic ester from the diacetone-

[20] Raymond, A. L., J. Biol. Chem., 83, 611 (1929).

glucose and the control have the same rates, while the Robison, and the synthetic ester from the monoacetoneglucose are much faster. The Robison starts a little sooner but the rates of the Robison and the synthetic are subsequently the same.

"Ale" Zymin.—In the fermenting mixtures the arsenate was 0.004 molal, the phospho-esters 0.06 molal. As the Robison ester removed the induction period to a certain extent with this zymin, hexosediphosphate, 0.01 molal, was added to the synthetic esters and only 0.005 molal to the Robison ester. It is seen in Fig. 4 that the Robison and the synthetic ester from the monoacetoneglucose ferment at about the same rate and much faster than the control. The synthetic ester from the diacetoneglucose on the other hand was appreciably inhibiting, a phenomenon occasionally observed in the case of this ester.

<div align="center">SUMMARY.</div>

1. Evidence is presented which substantiates the view that the synthetic monophosphate prepared through diacetoneglucose is glucose-3-phosphate. The Robison ester is shown to be not identical with this synthetic ester and therefore not glucose-3-phosphate.

2. An ester which is similar in many of its properties to the Robison ester has been synthesized through monoacetoneglucose. Its osazone is identical with that of the Harden-Young ester and it is therefore presumably glucose-6-phosphate. The possibility that the Robison ester is glucose-6-phosphate is discussed and further work is outlined.

THE NATURE OF THE SUGAR IN FOUR CASES OF PENTOSURIA.*

A CORRECTION.

By ISIDOR GREENWALD.

(From the Littauer Pneumonia Research Fund, New York University, New York.)

Dr. C. S. Hudson has kindly called the writer's attention to the fact that, in following Fischer's nomenclature in the designation of the xyloses, he has not observed the current practise. As is indicated by the rotation of the osazone, the xyloketose of the urine is derived from what is now known as l-xylose and has the configuration:

$$\begin{array}{c} CH_2OH \\ | \\ CO \\ | \\ HCOH \\ | \\ HOCH \\ | \\ CH_2OH \end{array}$$
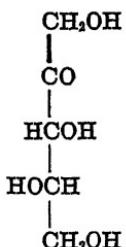
* Greenwald, I., *J. Biol. Chem.*, **88**, 1 (1930).

THE EFFECT OF WEAK ALKALIES ON THE TRIOSES AND ON METHYLGLYOXAL.

By H. A. SPOEHR and HAROLD H. STRAIN.

(*From the Carnegie Institution of Washington, Division of Plant Biology, Stanford University, California.*)

Most of the theories of carbohydrate catabolism postulate as a first step the splitting of the 6 carbon atom chain of the hexose molecule into 2 molecules containing 3 carbon atoms each. This conception arose first through the results obtained from a wide variety of purely chemical experiments with the hexose sugars (1–5). More recently this view has found some substantiation through the isolation of one of these intermediate products during biological carbohydrate catabolism. Thus methylglyoxal has been obtained from various biochemical reactions of the sugar molecule in both plants and animals (6–8). In this complicated succession of reactions and products the precursor of methylglyoxal and lactic acid is commonly assumed to be one of the trioses, glyceraldehyde or dihydroxyacetone.

In a quantitiative chemical study of the disintegration of the hexose molecule, either *in vivo* or *in vitro*, the following compounds, formed in the early stages of this action, must be considered: aldo- and ketohexoses, glyceraldehyde, dihydroxyacetone, the condensation products of these latter two, methylglyoxal, the condensation products of methylglyoxal, lactic acid, and probably also acetol and pyruvic acid. Since a thorough knowledge of these compounds and their mode of reaction under various conditions is essential for an understanding of the chemistry of carbohydrate metabolism, it would be highly desirable to have methods by means of which these compounds could be determined quantitatively in the presence of each other. Purely chemical methods have as yet not led to the desired goal, because of the great similarity of these compounds, the ease with which they are converted into each other, and their relatively high reactivity. This paper

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deals with some of the properties and reactions of the substances mentioned which may find application to the larger problem of carbohydrate transformations.

Glyceraldehyde.

Preparation (With William G. Young).—The best method for the preparation of *d,l*-glyceraldehyde is that developed by Wohl (9) and modified by Witzemann (10) and by Evans and Hass (11). The preparation is a rather difficult one, although if certain precautions are observed good results can be obtained. We were able thus to prepare over 400 gm. of a very satisfactory product. It may be helpful to mention a few points which need special care in the preparation.

Probably the greatest aid in the preparation of glyceraldehyde was that a good grade of stabilized acrolein could be purchased much more reasonably than it could be made in the laboratory.[1] An important precaution is necessary in the preparation of the β-chloropropionic aldehyde acetal. After the acrolein has been added to the alcoholic hydrogen chloride solution the β-chloropropionic aldehyde acetal separates out in a distinct layer. However, whether this is the upper or lower layer depends upon the degree to which the alcohol had previously been saturated with hydrogen chloride and, as a matter of experience, both layers contain considerable amounts of the β-chloropropionic aldehyde acetal. Therefore, it was found advantageous to allow the mixture to stand in an ice bath for at least an hour after all the acrolein had been added to the alcoholic hydrogen chloride. Then, without separating the layers, sodium bicarbonate was added to the mixture until it was neutral to litmus paper. The voluminous precipitate of sodium chloride was filtered off under strong suction and washed with a small amount of absolute alcohol. The filtrate consisted of two layers, the upper one being the β-chloropropionic ● aldehyde acetal. This was washed several times with water, separated, thoroughly dried with anhydrous sodium carbonate, and distilled at reduced pressure. Our average yield in this step was 55 per cent.

[1] From Les Établissements Poulenc Frères, 82 Rue Vieille-du-Temple, Paris, France.

The difficulty attending the conversion of β-chloropropionic aldehyde acetal to acrolein acetal has been commented upon by everyone who has published thereon. This difficulty is apparently due to the hygroscopic nature of the substances involved and the fact that the presence of water interferes with the desired course of the reaction. We have found that the relative humidity of the air has a decided influence on the success of the reaction. The best potassium hydroxide for the purpose was found to be a dry technical grade in lumps which could be rapidly ground to a very fine powder. It was necessary to observe every precaution to keep this material dry. In this step it was also found advantageous to place the powdered potassium hydroxide in an iron bomb which was cooled in an ice bath and then to add the ice-cold β-chloropropionic aldehyde acetal slowly and in small amounts while mixing thoroughly with the powdered potassium hydroxide. The bomb was then heated rapidly and the acrolein acetal distilled in the usual manner. Our yield of acrolein acetal was usually about 65 per cent.

For the formation of the glyceraldehyde acetal our experience corresponded with that given by Evans and Hass and our yields were about 60 per cent. Also in the hydrolysis of the glyceraldehyde acetal to glyceraldehyde it was found that the latter crystallizes from water if the vacuum distillation was carried out at a temperature not above 30°. In this step our average yields were 77 per cent. In our first preparations the melting point of the glyceraldehyde was 135° and 138°; later, after keeping the product in a vacuum over calcium chloride and soda-lime, it melted at 141–142°. Some of these preparations have been kept 4 years without deterioration.

Oxidation in Cuproalkaline Solution.—Nef ((2) p. 324) found that glyceraldehyde in solution with copper sulfate (2 mols) and sodium hydroxide (5 mols) was oxidized to glyceric acid when heated above 45°. Dihydroxyacetone under similar conditions yielded no glyceric acid.

With Benedict's solution (12) we found that glyceraldehyde had a reducing power 1.145 times that of glucose. Hence the oxidation of 1 mol of glyceraldehyde by this method required 1.68 atoms of oxygen, while glucose required 2.93 atoms.

Oxidation with Iodine.—By the method of Cajori (13) consistent

results corresponding to the quantitative oxidation of glyceralde-
hyde to glyceric acid were obtained. By allowing the mixture to
stand at room temperature for 25 minutes over 99 per cent of the
aldehyde had been oxidized; longer periods of time increased this
but very slightly. Glyceric acid is not oxidized by iodine under
the conditions used above. Salts such as sodium bicarbonate and
disodium phosphate did not promote the oxidation of glyceralde-
hyde by iodine solution.

Reduction of Phosphotungstomolybdic Acid.—The reduction by
glyceraldehyde was determined by the method which Campbell

TABLE I.

Reduction of Phosphotungstomolybdic Acid by Various Carbohydrates.

	Amount used.	Time of heating.	0.01 N KMnO₄ reduced.	1.0 N KMnO₄ equivalent to 1 gm. sugar.
	mg.	*min.*	*cc.*	*cc.*
Dihydroxyacetone.............	1.994	15	11.94	59.9
Glyceraldehyde...............	1.550	15	9.01	58.1
Mannose.....................	11.29	15	0.41	0.364
Glucose.....................	5.96	15	0.17	0.286
Galactose...................	6.60	15	0.19	0.288
Fructose....................	10.03	15	8.80	8.77
"	10.03	30	21.50	21.40
Xylose......................	5.83	15	0.58	10.00
Arabinose...................	6.46	15	0.45	6.99
Maltose.....................	8.85	15	0.10	0.113
Sucrose.....................	7.74	15	3.11	4.02
"	7.74	30	9.64	12.50

(14) suggested for the determination of dihydroxyacetone. The
results of determinations made in duplicate together with some
obtained with related compounds are given in Table I. 5 cc. of
the carbohydrate solutions were used with 5 cc. of the reagent and,
the mixtures were heated in a boiling water bath.

The results given in Table I show that glyceraldehyde has about
the same reducing power toward phosphotungstomolybdic acid as
dihydroxyacetone and that fructose has a very appreciable reduc-
ing power. The relatively high values for sucrose are no doubt due
to hydrolysis by the acid in the reagent. These results also show
that the method cannot be used for the determination of trioses

in the presence of pentoses, ketohexoses, or polysaccharides which are hydrolyzed to ketoses.

The phloroglucinol addition product of glyceraldehyde was described by Wohl and Neuberg (9) and has been used for the detection of glyceraldehyde. We have found, however, that with solutions which were more dilute than about 0.4 per cent the method is not applicable to quantitative determinations.

Catalytic Hydrogenation.—Glyceraldehyde is completely reduced by means of the Adams (15) platinum catalyst and hydrogen. Thus 0.10 gm. of glyceraldehyde in water with 0.01 gm. catalyst was completely reduced in 4 hours. Complete reduction occurred in the presence of 5 per cent disodium phosphate; in neutral phosphate solution 14 hours were required; in 1 per cent acetic acid solution the reduction was incomplete after 8.5 hours.

Conversion into Methylglyoxal.—Glyceraldehyde is readily converted into methylglyoxal in dilute acetic acid solution in the presence of amines at room temperature. This constitutes a fairly accurate means of determining glyceraldehyde, for in the presence of *m*-nitrobenzhydrazide the glyceraldehyde is quantitatively converted into the slightly soluble *m*-nitrobenzoylosazone of methylglyoxal. The reaction is described in detail in the accompanying paper.[2]

Dihydroxyacetone.

Autocondensation.—The dihydroxyacetone first used by us was obtained by recrystallization of oxantin (Höchst). Dihydroxyacetone undergoes spontaneous condensation very readily, a phenomenon which has recently been investigated by Levene and Walti (16); most of these findings we have confirmed. The oxantin, which was a dry white, crystalline material, was extracted with cold acetone, filtered, and dried in a vacuum. It melted at 80–81°. This was recrystallized either from absolute alcohol or from alcohol and acetone and yielded pure white crystals melting at 80–81°. After the oxantin had stood in the laboratory for a year it was found impossible to purify it in this manner; recrystallized dihydroxyacetone melting at 80°, after standing for a year melted at 75°.

[2] Strain, H. H., and Spoehr, H. A., *J. Biol. Chem.*, **89**, 527 (1930).

Oxidation in Cuproalkaline Solution.—The reducing power of dihydroxyacetone has been determined by the use of different methods and the values obtained differ, as compared to glucose, with different methods used. For example, Virtanen and Bärlund (17), using presumably monomeric dihydroxyacetone, m.p. 65–71°, with the Bertrand method, found 10 mg. had an equivalent of 7.9 mg. of copper. Bernhauer and Schön (18), using dimolecular dihydroxyacetone, m.p. 74–77°, with the Bertrand method, found that 10 mg. gave an equivalent of 13.05 mg. of Cu. The corresponding value for 10 mg. of glucose is 20.4 mg. of Cu (19). Levene and Blanco (20) state that "the monomeric form of dihydroxyacetone had a reducing power of 160 as compared with glucose (100)" with the Hagedorn-Jensen method. Fischler and Hirsch (21), with the same method and using oxantin, found this ratio to be 100:136. A method for the determination of dihydroxyacetone on the basis of the time required to reduce Fehling's solution in the cold has been described by Schmalfuss (22).

We found that dihydroxyacetone, m.p. 80–81°, had a reducing power toward Benedict's solution, with the centrifuge method described by Spoehr (12), of 1.11 times that of glucose, and hence required for oxidation 1.63 atoms of oxygen. The comparative reduction of phosphotungstomolybdic acid by dihydroxyacetone is shown in Table I.

Oxidation with Iodine.—Aldo- and ketohexoses can be readily differentiated on the basis that the former are oxidized by iodine while the latter are not (Cajori (13)). This method can, however, not be used to differentiate between glyceraldehyde and dihydroxyacetone as both of these compounds are oxidized by iodine. Glyceraldehyde is rapidly and quantitatively oxidized to glyceric acid, while with dihydroxyacetone the reaction is slow, but large quantities of iodine are reduced, indicating that the carbon chain is split with the formation of smaller molecules of high reducing power. In substantiation of this it was found that dihydroxyacetone is oxidized by bromine in water solution containing sodium carbonate, yielding glycolic acid. This was identified by analysis of its calcium salt. The other products formed in this oxidation were not identified. From 4.5 gm. of dihydroxyacetone in 100 cc. of water, oxidized with 20 gm. of bromine and 26 gm. of Na_2CO_3, there was obtained 0.15 gm. of pure anhydrous calcium glycolate.

On analysis 0.1332 gm. yielded 0.0392 gm. of CaO or 21.0 per cent of Ca; the theory for Ca $(C_2H_3O_3)_2$ is 21.05.

With the iodine solution of Cajori a definite end-point of the oxidation of glyceraldehyde is reached within a few minutes. Solutions of dihydroxyacetone, on the other hand, continue to react with iodine slowly for a long time, no definite end-point being reached. Attempts to modify the Cajori method by using sodium bicarbonate or disodium phosphate in place of sodium carbonate did not give satisfactory results. In this connection it is interesting that Nef ((2) p. 325) found that when dihydroxyacetone was oxidized with alkaline copper hydroxide no glyceric acid was formed, as was the case with glyceraldehyde under the same conditions.

Catalytic Hydrogenation.—Dihydroxyacetone is readily reduced by means of the Adams (15) platinum catalyst and hydrogen. In water solution 0.15 gm. and one-tenth the amount of catalyst required about 3 hours for complete hydrogenation. In solution with disodium phosphate about 4 hours were required and in neutral phosphate about 8 hours, while in acetic acid solution the reduction was very slow. Hydrogenation also took place in glacial acetic acid. That the dihydroxyacetone is thus reduced to glycerol was shown by converting the product of catalytic reduction to the benzoyl compound, m.p. 73°.

Methylglyoxal.

Preparation.—Although a number of methods have been described for the preparation of methylglyoxal, it is very doubtful whether any of these yields a pure product. Unfortunately in many experiments with methylglyoxal, both chemical and biological, this fact has not been given due consideration nor has the nature of the impurities been determined. From the work of Meisenheimer (23), the method of Wohl and Lange (24), in which the methylglyoxal diethylacetal is hydrolyzed, yields a fairly pure product, but it is not a convenient method for the preparation of larger quantities. The method of Fischer and Taube (25), which consists in distilling a mixture of dihydroxyacetone and phosphorus pentoxide at reduced pressure, is very simple, but required a supply of dihydroxyacetone and can be used only with small amounts, so that the maximum yields are about 5 gm. With this method there

is also the danger that the methylglyoxal may be contaminated with monomolecular dihydroxyacetone (25). We therefore used the method described by Denis (26) with some modifications. Although we finally obtained a product which contained only a very small amount of acid, it consisted of about 80 per cent methylglyoxal on the basis of the analytical methods employed.

In the Denis method, acetol in water solution is oxidized with copper acetate by allowing the mixture to stand at room temperature for several weeks. The acetol was prepared according to the method of Nef ((2) p. 247) which regularly gave a yield of about 60 per cent. In accordance with the warning of Nef the acetol was stored with an equal volume of methyl alcohol in order to prevent its condensation. However, when the acetol was pure we did not find that it was as susceptible to autocondensation as would be inferred from Nef's description.

For the preparation of methylglyoxal the acetol was dissolved in 7.3 times its weight of water and 5.4 gm. of copper acetate were added for each gm. of acetol. Cuprous oxide was soon precipitated and the solution became a deep purple color. After standing 3 to 4 weeks the cuprous oxide was filtered off under a vacuum and the slight excess of copper in solution was removed as rapidly as possible in the cold with hydrogen sulfide. The filtrate from the copper sulfide was distilled at reduced pressure at 35°. It was found later that the solution could be evaporated at reduced pressure and the residue extracted without previous treatment with hydrogen sulfide. The residual gum was taken up in dry ether and the solution dried thoroughly for a week with calcium chloride with the addition of a few gm. of calcium carbonate. The ether was then carefully distilled, the last portions being removed at reduced pressure at ordinary temperature. The flask containing the residual gum was kept in a vacuum desiccator over KOH for several days in order to remove the acetic acid remaining in the gum. This was then fractionally distilled in a vacuum, the receiver being cooled with solid carbon dioxide. The methylglyoxal collected in the bright yellow-green, mobile, monomolecular form, which on standing at 2-5° went into a thick colorless glass. When the latter conversion occurred at room temperature an appreciable evolution of heat was noticeable. This gum was soluble in water, though there was usually a small amount insoluble in water, which

in all probability was a polymer of methylglyoxal, as it formed the characteristic phenylosazone when heated with phenylhydrazine. When distilled in a vacuum the gum formed monomolecular methylglyoxal which is completely soluble in water.

Determination of Methylglyoxal.—The methods for determining methylglyoxal are not very satisfactory, especially in cases where carbohydrates or related substances are present. Comparative values obtained with a number of existing methods are given later in this section. Because of the fact that the possible contaminations of methylglyoxal may be closely related substances (*e.g.* lactides), analysis by combustion may not give definite information regarding the purity of the preparation.

The insoluble phenylosazone, m.p. 148°, the *p*-bromophenylosazone, which sinters at 184°, and the *p*-nitrophenylosazone, m.p. 280°, have been most generally used for the identification of methylglyoxal and are easily formed in dilute acid solution. The disemicarbazone, m.p. 254°, is also quite insoluble.

A wide variety of compounds was experimented with in the endeavor to find reagents for the determination of methylglyoxal with a view to differentiating quantitatively between this compound and the trioses and hexoses.

We have found that *m*-nitrobenzhydrazide and methylglyoxal form a very insoluble *m*-nitrobenzoylosazone of methylglyoxal. *m*-Nitrobenzhydrazide does not form an insoluble compound with glyceraldehyde and reacts only slowly with dihydroxyacetone. The compound formed with dihydroxyacetone is identical with the one formed with methylglyoxal. A description and analysis of this compound are given in the paper which follows.[2] An idea of the solubility of this compound can be gained from the following. A solution of methylglyoxal containing 0.54 mg. of methylglyoxal per 100 cc. formed a precipitate after standing 25 minutes, while a solution containing 0.054 mg. per 100 cc. did not form a precipitate after standing 16 hours. *m*-Nitrobenzhydrazide also possesses the advantage that it does not form slightly soluble compounds with phosphates or with hexoses.

Because of the uncertainty regarding the purity of most preparations of methylglyoxal there exists some doubt as to the reliability of the methods of analysis which have been developed by the use of such preparations. We have therefore made comparative

analyses by the use of several methods involving different principles. The results throw some light on the reliability of the methods in question. The methods used were (1) that described by Friedemann (27), (2) that of Fischler and Boettner (28), and (3) the gravimetric determination as the p-bromophenylosazone, the p-nitrophenylosazone, and the m-nitrobenzoylosazone. The method of Kuhn and Heckscher (29) was not used, because as these authors state this method gives only comparative values.

The solution, Solution A, 200 cc., contained 3.92 gm. by weight of methylglyoxal prepared by the modified Denis method. This contained 0.052 gm. of acetic acid as determined by titration with

TABLE II.

Analysis, by Different Methods, of Solutions of Methylglyoxal.

Under Solution A are the results obtained with a solution containing 3.92 gm. of methylglyoxal in 200 cc. of solution. Under Solution B are the results obtained with the distillate from the treatment of glyceraldehyde with sulfuric acid, in per cent of the theoretical quantity of methylglyoxal.

	As lactic acid with excess of N NaOH.	Friedemann's method, NaOH + H_2O_2.	m-Nitro-benzhydrazide.	p-Nitro-phenylhydrazine.	p-Bromo-phenylhydrazine.	Fischler and Boettner's method.
	per cent	per cent	per cent	per cent	per cent	per cent
Solution A....	76.65±0.1	80.63±1.7	81.3±2.1	79.6±1.6		78.5±0.3
" B....		81.70±0.5	84.8	84.4	83.3	83.1±0.2

0.1 N NaOH in the cold. A portion of the solution was heated on the boiling water bath for 5 minutes with an excess of 1.0 N NaOH, cooled, and the excess titrated with 1.0 N HCl in order to determine the methylglyoxal as lactic acid. In Table II, under Solution A, are given the results of the different methods of analysis of this solution.

Another solution of methylglyoxal, Solution B, which had been prepared by distilling glyceraldehyde with sulfuric acid according to Neuberg, Färber, Levite, and Schwenk ((30) p. 264), was analyzed by the same methods. Glyceraldehyde, 1.3126 gm., was added to a solution of 2.6 cc. of concentrated sulfuric acid in 13 cc. of water and the mixture distilled; 5 cc. of water were added to the distilling mixture as 5 cc. portions distilled over. The distillate

was slightly acid; the titration value, calculated as acetic acid, amounted to 0.026 gm.

The different methods do not show very satisfactory agreement; whether this is due to inherent faults in the methods or to the fact that the preparations of methylglyoxal contained some impurity, these experiments cannot reveal. Determinations by conversion into lactic acid with an excess of alkali are somewhat low; this may indicate that under these conditions some of the methylglyoxal undergoes condensation, as is discussed later, rather than being

TABLE III.

Determination of Methylglyoxal (3.92 Gm in 200 Cc. of Solution)
Solution A.

This solution was diluted 5 times and analyzed by the method of Fischler and Boettner; in each case 30.18 cc. of 0.1 N iodine solution were used and the oxidation time was ½ hour at 20°.

Methylglyoxal solution.	N NaOH used.	N HCl to neutral-ize excess alkali.	0.1 N iodine con-sumed.	Methylglyoxal found.
cc.	*cc.*	*cc.*	*cc.*	*per cent*
2	5	7.5	6.83	78.4
2	10	12.5	6.82	78.3
2	20	25	6.83	78.4
4	20	25	13.61	78.1
5	5	7.5	14.56	66.8
5	10	12.5	16.08	73.7
5	20	25	17.15	78.8
6	20	25	20.48	78.3
6	20	25	20.51	78.5
8	20	25	25.00	71.7
8	20	25	26.88	77.1

converted quantitatively into lactic acid. Our experience with the method of Fischler and Boettner (28) has shown that it is highly important that a decided excess of both alkali and iodine be used in order to obtain consistent results. This fact is not stressed by Fischler and Boettner, but is demonstrated in the results shown in Table III giving the analyses of Solution A with varying ratios of methylglyoxal, alkali, and iodine. Separate experiments with this method demonstrated that the same values were obtained when an oxidation period of 1 hour was used instead of a period of ½ hour.

Oxidation in Cuproalkaline Solution.—Owing to the fact that methylglyoxal has not been obtained in pure crystalline form its reducing power is still uncertain. On the basis of determinations of the amount of methylglyoxal present, determined as the phenylosazone or *p*-nitrophenylosazone in various samples, it was found that the reducing power toward Benedict's solution was about 30 per cent that of glucose, though this value showed considerable variation with different preparations.

Effect of Weak Alkalies on Glyceraldehyde.—It has been very generally assumed that in the splitting of glucose glyceraldehyde (or dihydroxyacetone) is the precursor of methylglyoxal and that this in turn is the precursor of lactic acid. Owing to the complexity of the chemistry of these reactions and because of the theoretical possibility of the mutual interconversion of these compounds, it is highly important that such deductions should be carefully scrutinized on the basis of quantitative data, if valid conclusions are to be obtained. It has unfortunately been impossible as yet to follow all these reactions with anything approaching quantitative precision even *in vitro.*

The formation of lactic acid from glyceraldehyde by the action of alkali has been reported by a number of workers; *viz.,* Nef ((2) p. 329), Evans and co-workers (11, 31), Bernhauer and Schön (18). It is noteworthy that in these experiments the amounts of lactic acid formed were small, in spite of the fact that the concentration of alkali used was high. Oppenheimer (32) also reported that with N sodium hydroxide at 37° about 50 per cent of the glyceraldehyde is converted into lactic acid, while with 0.1 N NaOH only a very small amount of lactic acid was formed. Similarly, when glyceraldehyde is treated with strong alkalies in the presence of phenylhydrazine, the osazone of methylglyoxal is precipitated. With N potassium hydroxide at 50° Evans and his collaborators (11, 31) found that about 27 per cent of the glyceraldehyde is converted into methylglyoxal; while with 0.2 N alkali about 12 per cent of this conversion occurred.

In our experiments with disodium phosphate and sodium carbonate there is no direct evidence of the formation of methylglyoxal from glyceraldehyde, although under these conditions, the latter undergoes decided changes.

Glyceraldehyde, 1.200 gm., and 10.0 gm. of $Na_2HPO_4 \cdot 12H_2O$

were dissolved in 480 cc. of water, 2 cc. of xylene were added, and the solution kept in a stoppered flask in a thermostat at 37°. At intervals samples were withdrawn and the reducing power toward Benedict's solution (12) and iodine (13) was determined. The same determinations were made on a solution of glyceraldehyde in water containing neutral phosphate. A condensed summary of the results is given in Table IV. With time the glyceraldehyde disappears rapidly and both the copper and the iodine reducing values diminish. Pure glyceraldehyde when kept in aqueous solution at 37° for 8 days shows no change in reducing power toward Benedict's solution or iodine. Analogous experiments were also

TABLE IV.

Effect of Disodium Phosphate (Solution A) and Neutral Phosphate Mixture (Solution B) on Glyceraldehyde As Indicated by Change in Reducing Power toward Benedict's Solution and toward Iodine.

Time.	Reduction of Benedict's solution, calculated as glyceraldehyde.		Reduction of iodine, calculated as glyceraldehyde.	
	Solution A.	Solution B.	Solution A.	Solution B.
hrs.	gm.	gm.	gm.	gm.
0.5	1.173	1.247	1.174	1.085
24	1.026	1.168	0.425	0.766
48	0.951	1.135	0.359	0.720
96	0.831	0.898	0.284	0.662
192	0.685	0.741	0.249	0.612
384	0.540		0.251	
768	0.371		0.167	
1536	0.267			

carried out with dihydroxyacetone. These and the ones with glyceraldehyde are discussed later in this paper.

Similar results were obtained by the use of sodium carbonate. In these more strongly alkaline solutions the glyceraldehyde disappears much more rapidly, so that 85 per cent of the glyceraldehyde as determined by iodine had disappeared in 16 hours.

A noteworthy fact in the formation of methylglyoxal from hexoses by the action of alkalies according to the procedure of Nef ((2) p. 254), Dakin and Dudley (3), and of Neuberg and Oertel (4) is that the methylglyoxal cannot be obtained unless it is immediately removed from the solution or bound by some such reagent

as phenylhydrazine with which it forms an insoluble osazone. In other words, the methylglyoxal does not accumulate, but on account of its great reactivity rapidly undergoes further changes. We shall refer to this again under the experiments with methylglyoxal. In order to determine whether any methylglyoxal was formed from glyceraldehyde when this was treated with disodium phosphate, 1 gm. of glyceraldehyde was dissolved in a solution containing 0.5 gm. of $Na_2HPO_4 \cdot 12H_2O$, 1.11 cc. of phenylhydrazine, 1.11 cc. of alcohol, and 5.56 cc. of water. The mixture stood for 42 days in a thermostat at 25°, but not a trace of methylglyoxal phenylosazone was formed.

It is somewhat difficult to understand the experiments of Fischler (33, 34) who claims to have demonstrated that glyceraldehyde and dihydroxyacetone are intermediate products in the formation of methylglyoxal from glucose when a solution containing this sugar, sodium carbonate, and sodium bisulfite is distilled in a stream of carbon dioxide. In the residual solution from this distillation Fischler states, there is contained glyceraldehyde or dihydroxyacetone, which, it is stated, was identified as the phenylosazone. In the paper of Fischler and Lindner ((34) p. 246), however, it is stated that dihydroxyacetone (oxantin) is much more sensitive to this reaction mixture.

"Es werden also relativ grosse Mengen von Dioxyaceton unter unserer Versuchsordnung in kurzer Zeit so weitgehend verändert, dass eine Osazonbildung nicht mehr eintritt." "Während es leicht gelingt das Dioxyaceton-osazon aus dem reinen Oxantin 'Höchst' darzustellen, gelingt dies nicht mehr, wenn man Oxantin auch nur sehr kurze Zeit in der von uns angewendetenWeise aus alkalischem Medium destilliert."

The same results were obtained with a crude preparation of glyceraldehyde. If these trioses are so very sensitive to alkali how could they be detected in such a mixture which had been boiled for an hour?

Glyceraldehyde is converted very readily and quantitatively into methylglyoxal by means of certain primary amines in acid solution. The conditions under which this reaction takes place and its application to the determination of methylglyoxal are described in the following paper.[2]

Effect of Weak Alkalies on Dihydroxyacetone.—Neuberg and Rewald (35) were able to demonstrate the formation of methyl-

glyoxal from dihydroxyacetone when the latter is heated in a solution containing 4 per cent of sodium carbonate and phenylhydrazine.   We have been able to confirm this by allowing a solution containing 5 gm. of dihydroxyacetone, 5 gm. of Na_2CO_3, 15 cc. of phenylhydrazine, 15 cc. of alcohol in 60 cc. of water to stand in a thermostat at 25°.   Evans and Cornthwaite (31) obtained about a 4 per cent conversion with 0.2 N KOH at 25°.

However, with the more weakly alkaline disodium phosphate, under the same conditions, there was formed only glycerosephenylosazone.   From 0.5 gm. of dihydroxyacetone, 0.55 gm. of $Na_2HPO_4 \cdot 12H_2O$, 1.11 cc. of phenylhydrazine, and 1.11 cc. of

TABLE V.

Effect of Disodium Phosphate on Dihydroxyacetone at 37° As Indicated by Change in Reducing Power toward Benedict's Solution and Iodine.

Time.	Reduction of Benedict's solution, calculated as dihydroxyacetone.	Reduction of 0.1 N iodine per gm. dihydroxyacetone.
hrs.	*gm.*	*cc.*
0.5	1.197	53.0
24	1.130	68.5
48	0.994	80.3
96	0.818	80.3
192	0.556	70.1
384	0.318	63.2
768	0.190	42.4

alcohol in 5.56 cc. of water there were obtained, after allowing the mixture to stand for 83 days at 25°, 0.12 gm. of glycerosephenylosazone, melting at 130° and showing on analysis 20.75 and 20.63 per cent of nitrogen, the theory for $C_{15}H_{16}ON_4$ being 20.90 per cent. The same compound was obtained in a shorter time at 37°.

As is the case with glyceraldehyde, the main action of a weak alkali, such as disodium phosphate, is of a complex nature.   In Table V is shown the effect of disodium phosphate on the reducing power toward Benedict's solution and iodine of a solution of dihydroxyacetone.   This solution contained 1.200 gm. of dihydroxyacetone, 10 gm. of $Na_2HPO_4 \cdot 12H_2O$, and 2 cc. of xylene in 480 cc. of water and was kept at 37°.

Both of the trioses, glyceraldehyde and dihydroxyacetone, are

exceedingly sensitive to the action of alkali. With higher hydroxyl ion concentrations and at higher temperatures the trioses are converted to methylglyoxal and lactic acid, although even with 6 N KOH and at 50° Evans and Cornthwaite (31) obtained less than 45 per cent of lactic acid and with 0.2 and 0.4 N KOH none was formed. At lower hydroxyl ion concentrations, as exist in solutions of disodium phosphate, the trioses are also drastically affected, but with the formation of other products. This change is accompanied by a decided decrease in the reducing power of the triose solutions toward both Benedict's solution and iodine.

The suggestion of Nef (36) that in solution with weak or dilute alkalies the monosaccharides undergo interconversion to form a restricted equilibrium ("beschränkte Gleichgewichtsverhältnisse"), consisting of a mixture of various aldo- and ketomonosaccharides, has been followed by numerous workers in the theoretical discussions of the effect of alkalies on sugars. It seems very doubtful, however, whether such a conception can be applied to the trioses in disodium phosphate or neutral phosphate solution. The results on the reducing power toward Benedict's solution and iodine clearly show that there is no such equilibrium between glyceraldehyde and dihydroxyacetone; i.e., that here we are not dealing with a simple aldo-keto interconversion. The decided decrease in the reducing power toward iodine of both the glyceraldehyde and dihydroxyacetone mixtures might be taken to indicate a condensation to ketohexoses as was found by Schmitz ((37) p. 2332), for ketoses do not reduce iodine. On the other hand, if ketohexoses were present there would not be such a decided decrease in the reducing power toward Benedict's solution. That there were no appreciable quantities of aldohexoses present is indicated by the low reducing powers toward both Benedict's solution and iodine. It has, moreover, previously been shown (Spoehr and Strain (38)) that the effect of disodium phosphate on the hexoses is very slow. It has also been shown that under these conditions neither glyceraldehyde nor dihydroxyacetone is converted into methylglyoxal or lactic acid.

In all probability glyceraldehyde and dihydroxyacetone in solution with disodium phosphate or neutral phosphate mixtures undergo condensations of a complex and yet unknown nature. This is further indicated by the fact that although the reducing

power toward iodine, on the basis of the Cajori method, had decreased decidedly, at the end of the experiments the reaction mixtures took up, though slowly, large quantities of iodine. This was made evident when the oxidation period of the Cajori method was extended from 25 minutes to 1 hour.

Effect of Weak Alkalies on Methylglyoxal.—That methylglyoxal is easily converted into lactic acid by means of stronger alkalies or by simply heating an aqueous solution has been known for a long time (Denis (26)). However, in weakly alkaline solution, pH 9.0 to 10.5, while the methylglyoxal also disappears, it is not converted quantitatively into lactic acid. This fact has also been reported by Ariyama (7), who noted that at these hydrogen ion concentrations "the appearance of lactic acid falls far behind the amount of methylglyoxal which disappeared," and that the conversion is quantitative only at higher alkalinity, pH 12.5, while "the fate of methylglyoxal at lower hydrogen ion concentration is unknown. . . ."

A solution of 2.17 gm. of methylglyoxal and 44.8 gm. of $Na_2HPO_4 \cdot 12H_2O$ in 250 cc. of water (0.5 M) was kept in a thermostat at 25° and determinations of reducing power and methylglyoxal made at intervals. The reducing power toward Benedict's solution increased rapidly, so that after 24 hours it had increased by about 15 per cent. This increased reducing power of the methylglyoxal-phosphate solution remained practically constant for 12 days and longer. At the same time the amount of methylglyoxal present in the solution, as determined with m-nitrobenzhydrazide, decreased very rapidly during the first 24 hours. As the condensation product of methylglyoxal forms a slightly soluble compound with m-nitrobenzhydrazide it was necessary to dry the precipitates, extract with warm ethyl acetate which dissolves the precipitated condensation product compound, and finally to dry and weigh the m-nitrobenzoylosazone. That the m-nitrobenzoylosazone of methylglyoxal is insoluble in ethyl acetate is shown by the fact that 0.4106 gm. of this material lost less than 0.1 mg. when thoroughly washed with warm ethyl acetate (20 cc.). In Fig. 1 the curves are reproduced showing the increase in reducing power and the decrease in the amount of methylglyoxal present in the solution. Similar results were obtained with sodium carbonate used in place of disodium phosphate. In this case there was a

more rapid increase in the reducing power, which was not as great as that observed with phosphate and which slowly diminished, indicating the possible formation of small amounts of lactic acid. In a special experiment this was in fact found to be the case. At 37° methylglyoxal-phosphate mixtures also show a decrease in the reducing power after the initial increase which requires 96 hours, with the formation of small amounts of lactic acid.
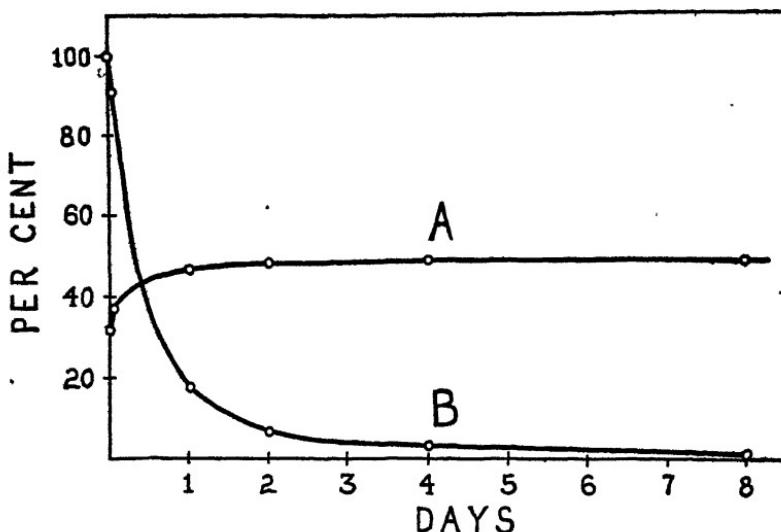
FIG. 1. Effect of disodium phosphate on a solution of methylglyoxal at 25°. Curve *A* indicates the change in reducing power toward Benedict's solution as per cent that of glucose. Curve *B* indicates the change in methylglyoxal content of the solution as per cent of the quantity originally present.

Condensation of Methylglyoxal by Weak Alkalies.—It has not been possible to establish definitely the nature of the condensation products which are formed from methylglyoxal by the action of weak alkalies. Methylglyoxal is undoubtedly capable of reacting in a variety of ways; in all probability it is this fact which makes it important as an intermediate compound in carbohydrate catabolism. That the condensation product obtained from methylglyoxal with weak alkalies at ordinary temperatures does not

contain much triose is indicated by the fact that the reducing power toward Benedict's solution of the condensation product does not change appreciably in disodium phosphate solution (Fig. 1), while both glyceraldehyde and dihydroxyacetone under these conditions show a decided and rapid decrease in reducing power, as was pointed out in a previous portion of this paper.

The following facts are of significance in a consideration of the nature of this condensation product; they indicate that it is composed to an appreciable extent of $CH_3 \cdot C:O-$ groups; that is, that the methylglyoxal has not undergone changes, to a very appreciable degree, of the nature of the conversion into trioses.

A fresh preparation of methylglyoxal, 16.6 gm., was dissolved in water to make 200 cc. of solution. This solution was analyzed for methylglyoxal by several methods. Thus by the determination with m-nitrobenzhydrazide the preparation contained 68.3 per cent methylglyoxal, by Friedemann's method 68.7 per cent, and by Fischler and Boettner's method 67.2 per cent; the reducing power of the methylglyoxal was 10.7 per cent that of glucose. To the remainder of the solution, 185 cc., 28.4 gm. of disodium phosphate were added. The tightly sealed solution was allowed to stand 5 days at 37°. The reducing power toward Benedict's solution now showed a decided increase; i.e., 27.8 per cent that of glucose calculated on the same basis as above. However, the methylglyoxal present, as determined with m-nitrobenzhydrazide, had decreased very appreciably and showed that there were in the solution only 0.097 gm. of methylglyoxal.

The method of analysis of Friedemann and that of Fischler and Boettner are, of course, not specific for methylglyoxal. This is shown by the fact that the solution of methylglyoxal which had been treated with disodium phosphate showed on analysis by Friedemann's method 2.19 gm. of methylglyoxal and by Fischler and Boettner's method 5.76 gm. It should be mentioned that while in the original solution of methylglyoxal the duplicate analyses agreed well, in the final methylglyoxal-phosphate solution the analytical results were variable.

The methylglyoxal-phosphate solution was evaporated under reduced pressure and the residue extracted with absolute alcohol. The alcohol was then evaporated under reduced pressure, the residue dissolved in water, and the solution in turn evaporated in

the same way. This distillate was found to contain a small amount of methylglyoxal, so the residue was again dissolved in water and evaporated; this distillate did not give a positive test for methylglyoxal with m-nitrobenzhydrazide. The residual gum, 12.0 gm., was dissolved in water to make 100 cc. of solution. A portion, 15 cc., of this solution was then distilled with sulfuric acid by the method of Neuberg et al. (30) for the determination of trioses by conversion into methylglyoxal. The first 100 cc. of the distillate were analyzed for methylglyoxal and it was found that by the use of m-nitrobenzhydrazide 1.02 per cent of the sample had been converted into methylglyoxal and by analysis with the method of Fischler and Boettner 1.85 per cent. This shows clearly that the condensation product obtained by the action of disodium phosphate on methylglyoxal could have contained only a very small amount of trioses. It is important to note that the last portions of the distillate gave positive tests for methylglyoxal with m-nitrobenzhydrazide. When trioses, even in much larger quantities, were treated in the same manner, the methylglyoxal was removed completely before 100 cc. had distilled over and yielded very nearly the theoretical amounts for the trioses used.

It was found that from the original methylglyoxal solution, when treated with 6.0 N sodium hydroxide and iodine there were obtained 3.025 gm. of iodoform per gm. of crude methylglyoxal. Neither of the trioses when thus treated formed iodoform. From the methylglyoxal condensation product when treated in this way there was obtained 0.93 gm. of iodoform per gm. Although this is not an accurate method of estimating methylglyoxal or the $CH_3 \cdot C:O-$ group, the experiment does throw some light on the nature of the condensation product.

When the methylglyoxal condensation product was tested for the presence of glyceraldehyde by treating the solution with aniline and m-nitrobenzhydrazide, no evidence of the presence of this triose was ever obtained.

The number of theoretically possible compounds which could be formed from methylglyoxal through an aldol or benzoin condensation is very great and in all probability the condensation product with which we are dealing here is not a single compound. It forms definite compounds with several phenylhydrazines, but repeated efforts did not lead to their identification. The yields

of these addition compounds are also very small. A solution of 8.9 gm. of methylglyoxal, 3 gm. of Na_2CO_3 in 200 cc. of water, was allowed to stand for 6 days at room temperature, when it no longer formed the characteristic phenylosazone of methylglyoxal. After adding 52 cc. of N hydrochloric acid, the solution was distilled in a vacuum at 35°. The distillate contained a small amount of methylglyoxal as was shown by the formation of the p-nitrophenylosazone. The residue was extracted with alcohol, filtered from the sodium chloride, and the alcohol evaporated in a vacuum, leaving 6.5 gm. of gum. To one-half of the aqueous solution of this gum was added very slowly a dilute solution of phenylhydrazine acetate. The crystalline precipitate, 1.5 gm., which was formed was very soluble in all organic solvents and could not be recrystallized. To the filtrate was added more phenylhydrazine acetate and the mixture was heated on the water bath. The tarry precipitate which formed was filtered and extracted with hot alcohol. From the alcoholic solution, on cooling, yellow crystals, 0.2 gm., separated which on recrystallizing from methyl alcohol several times had a constant melting point of 159–160° and contained, as shown by combustion (Pregl), 18.76 and 18.80 per cent of nitrogen. The other half of the solution of the condensation product of methylglyoxal when heated with p-nitrophenylhydrazine and acetic acid formed 1.7 gm. of an insoluble red compound. This could be purified only by dissolving in ethyl acetate and adding carbon tetrachloride. It did not melt sharply, but sintered at 140–160°. By combustion analysis (Pregl) 17.91 and 17.94 per cent nitrogen were obtained and the molecular weight, determined by boiling point rise in acetone, was about 500.

From the foregoing it is clear that the methylglyoxal disappears in a solution containing disodium phosphate. The reaction of the resulting product toward hydrazine derivatives indicates that there was present but a small amount of substances containing the $\cdot C:O\cdot CH:O$ grouping. At the same time the large amounts of iodoform which were obtained from the product indicate that the $CH_3\cdot C:O-$ or CH_3CHOH- groups are present. The fact that the reducing power toward Benedict's solution is higher than the original methylglyoxal solution indicates that but very little lactic acid was formed. If a conversion of methylglyoxal to trioses took place this could constitute only 1.5 per cent of the condensation

product, as was shown by the distillation method of Neuberg *et al.* Further proof of the absence of trioses in the condensation product is afforded by its reaction toward *m*-nitrobenzhydrazide and aniline.

As methylglyoxal undergoes condensation with dilute alkalies so readily, it is very probable that similar changes occur to some extent even with stronger alkalies, as is indicated by the results of Ariyama (7). Moreover, this behavior of methylglyoxal must be considered in the interpretation of all experiments designed to determine the intermediate steps in the breakdown of hexoses in alkaline and in netural salt solutions (Bernhauer and coworkers (39)).

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THE EFFECT OF AMINES ON THE CONVERSION OF TRIOSES INTO METHYLGLYOXAL.

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An investigation of the reactions of amines on the triose sugars was undertaken with the object of estimating and differentiating d,l-glyceraldehyde and dihydroxyacetone, particularly in the presence of methylglyoxal and the hexose sugars. This research led to the surprizing observation that many organic amines cause the formation of methylglyoxal from glyceraldehyde in dilute acetic acid solution. Subsequent experiments then demonstrated that glyceraldehyde was quite stable in dilute acetic acid even over long periods of time when no amines were present.

In order to determine the methylglyoxal formed as a result of the action of amines on glyceraldehyde it was necessary to procure a methylglyoxal reagent which was soluble in dilute acetic acid and which would not react with amines or with the trioses. Such a reagent was m-nitrobenzhydrazide.

When glyceraldehyde was permitted to stand with small quantities of aniline in dilute acetic acid solution, the methylglyoxal produced underwent further change so that the yields never amounted to much more than 50 per cent of the theoretical quantity. By carrying out this reaction in the presence of m-nitrobenzhydrazide the glyceraldehyde was quantitatively converted into the slightly soluble m-nitrobenzoylosazone of methylglyoxal. Similar use of m-nitrobenzhydrazide has permitted the study of the effect of many organic compounds on the conversion of glyceraldehyde into methylglyoxal.

Dihydroxyacetone in contradistinction to glyceraldehyde is slowly and incompletely converted into methylglyoxal in dilute acetic acid solution. The presence of amines and m-nitrobenzhydrazide has but little effect on the reaction.

As a consequence of these observations it is now possible to differentiate and to estimate glyceraldehyde and methylglyoxal in the presence of each other and the hexoses. This was done by first precipitating the methylglyoxal with *m*-nitrobenzhydrazide and filtering. The glyceraldehyde was then quantitatively converted into the *m*-nitrobenzoylosazone of methylglyoxal by adding aniline and *m*-nitrobenzhydrazide to the filtrates.

The results of these experiments and a description of the reactions and compounds involved are included in the experimental part.

<div align="center">EXPERIMENTAL.</div>

Methylglyoxal and m-Nitrobenzhydrazide.—A dilute solution of methylglyoxal reacts almost immediately with a dilute solution of *m*-nitrobenzhydrazide in dilute acetic acid to form a very slightly soluble, white, crystalline compound which is also only slightly soluble in the common organic solvents. Recrystallized from nitrobenzene and dried in the vacuum oven at 110–130° for 4 hours, the crystals decomposed at 280°. On analysis by combustion (Pregl) this material gave results agreeing with the *m*-nitrobenzoylosazone of methylglyoxal.

$C_{17}H_{14}O_6N_6$.   Calculated.   N 21.10.
              Found.   " 20.75, 20.77.

The slight solubility of methylglyoxal-*m*-nitrobenzoylosazone in dilute acetic acid makes this material very suitable for the quantitative estimation of methylglyoxal. (See the preceding article.[1])

Glyceraldehyde and m-Nitrobenzhydrazide.—Glyceraldehyde does not form an insoluble compound when permitted to stand with *m*-nitrobenzhydrazide in dilute acetic acid solution for 21 days. Heating the solution to boiling for 15 minutes does not cause the formation of a precipitate. On the other hand, if an amine such as aniline is added to the boiling solution, a white precipitate separates almost immediately, while if the amine is added to the solution at room temperature, the precipitate forms after a few minutes and then increases in quantity for several days. The

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[1] Spoehr, H. A., and Strain, H. H., *J. Biol. Chem.*, **89**, 503 (1930).

precipitate obtained by this reaction decomposed at 280° after recrystallization from nitrobenzene. Its decomposition point was not altered by mixing it with the m-nitrobenzoylosazone of methylglyoxal. The analysis further substantiates the identity of these compounds.

$C_{17}H_{14}O_6N_6$.   Calculated.   N 21.10.
                                 Found.   " 20.53, 20.52.

The rate of methylglyoxal formation from glyceraldehyde in the presence of aniline and m-nitrobenzhydrazide was followed at room temperature and at 25°. This was accomplished by adding 10 cc. portions of a standard glyceraldehyde solution to 100 cc. of m-

TABLE I.

Methylglyoxal-m-Nitrobenzoylosazone Formed from Glyceraldehyde in the Presence of Aniline and Acetic Acid at 25°.

Glyceraldehyde.	Time of reaction.	Methylglyoxal-m-nitrobenzoylosazone.	Theory.
gm.	hrs.	gm.	per cent
0.09383	24	0.2831	68.2
0.09383	48	0.3760	90.6
0.09383	96	0.4016	96.8
0.09383	192	0.4138	99.7
0.09383	336	0.4138	99.7
0.09383	480	0.4139	99.8

nitrobenzhydrazide (0.5 gm.) solutions each of which contained 1 cc. of aniline and 1 cc. of acetic acid. The solutions which were contained in glass-stoppered flasks were permitted to stand in the thermostat. From time to time flasks were removed, the solutions filtered, and the precipitates dried in a vacuum over sulfuric acid. The dry precipitates were then extracted with 10 to 20 cc. of warm ethyl acetate to remove any trace of adsorbed aniline acetate, again dried, and weighed. The results obtained at 25° are summarized in Table I. A control experiment in which no aniline was added formed only 0.0274 gm. of precipitate after 20 days. When aniline (1 cc.) was finally added and the solution permitted to stand 4 days, a precipitate weighing 0.3684 gm. was obtained, thus showing that at least 88.8 per cent of the glyceraldehyde was present after 20 days.

Dihydroxyacetone and m-Nitrobenzhydrazide.—Dihydroxyacetone reacts slowly and incompletely with a solution of *m*-nitrobenzhydrazide in dilute acetic acid forming a white crystalline compound which decomposes at 280° after recrystallization from nitrobenzene. The mixed melting point and the nitrogen content agreed with those of methylglyoxal-*m*-nitrobenzoylosazone.

$C_{17}H_{14}O_6N_6$.　Calculated. N 21.10.
　　　　　　　　Found.　" 20.97, 21.06.

The rate of methylglyoxal formation from dihydroxyacetone was followed both in the presence and in the absence of aniline. The procedure was the same as that described for glyceraldehyde.

TABLE II.

Methylglyoxal-m-Nitrobenzoylosazone Formed from Dihydroxyacetone in Acetic Acid Solution with and without Aniline, at 15–20°.

Dihydroxyacetone.	Time of reaction.	Catalyst.	Methylglyoxal-m-nitrobenzoylosazone.	Theory.
gm.	*days*		*gm.*	*per cent*
0.07002	9	None.	0.0585	19.0
0.07002	15	"	0.0956	30.8
0.07002	30	"	0.1316	42.4
0.07002	9	Aniline (1 cc.).	0.1320	42.6
0.07002	15	"　　(1 ").	0.1773	57.2

The total volume of the solution which contained acetic acid (1 cc.) and *m*-nitrobenzhydrazide (0.5 gm.) was 100 cc. The results are given in Table II.

Substances Which Aid Methylglyoxal Formation from Glyceraldehyde.—A number of substances were tested as catalysts for the conversion of glyceraldehyde into methylglyoxal in the presence of *m*-nitrobenzhydrazide. The observations were made in test-tubes by adding 2 cc. of a glyceraldehyde solution (0.02 gm. of glyceraldehyde) to 8 cc. of a saturated solution of *m*-nitrobenzhydrazide in dilute acetic acid containing approximately 0.2 gm. of the substance to be tested. The tubes were then permitted to stand at room temperature. The results of these tests are given in Table III.

Methylglyoxal and 4,4-Diphenylsemicarbazide.—Methylglyoxal

TABLE III.

Compounds Tested As Catalysts for Conversion of Glyceraldehyde into Methylglyoxal.

The substances which caused a rapid formation of methylglyoxal-*m*-nitrobenzoylosazone are indicated by ++, those which caused the slow formation of this compound by +, and those which caused no reaction in 72 hours by −.

Alanine	−	Ethylenediamine	+
p-Aminoacetanilide	++	Glycocoll	−
p-Aminobenzoic acid	+	" ethyl ester	−
2-Aminocymene	++	Guaiacol	−
4-Amino-1,3-dimethylbenzene	++	Guanidine	−
2-Amino-4-nitrophenol	++	Hydroxylamine	−
Ammonium acetate	−	Inositol	−
Amylamine	−	Leucine	−
Iso-amylamine	−	Lysine picrate	−
Iso-amylcarbamate	−	α-Naphthylamine	++
Aniline	++	β-Naphthylamine	++
p-Anisidine	++	3-Nitro-4-aminoanisole	−
Anthranilic acid	+	3-Nitro-4-aminotoluene	−
Arginine	−	*p*-Nitroaniline	+
Asparagine	−	Phenol	−
Benzidine	++	*o*-Phenylenediamine	−
Benzylamine	−	Phenylglycine ethyl ester	−
p-Bromoaniline	++	Phenylsemicarbazide	−
Brucine	−	Phloroglucinol	−
d-Camphoric acid	−	Picric acid	−
Capryl alcohol	−	Pinacone	−
m-Chloroaniline	++	Pyridine	−
p-Chloroaniline	++	Quinine	−
Citric acid	−	Resorcinol	−
Cyclohexylamine	−	Salicylic acid	−
Diaminoanisole	++	Semicarbazide	−
1,4-Diaminobutane	+	Tartaric acid	−
2,5-Diaminotoluene	++	Tartronic acid	−
Di-*n*-butylamine	−	*p*-Toluidine	++
Dimethylaniline	−	Tyrosine	−
Dimethylglyoxine	−	Urea	−
Diphenylamine	−	" nitrate	−
Ethylamine	−	Urethane	−
Ethyl-*p*-aminobenzoate	+	Uric acid	−

reacts with a solution of 4,4-diphenylsemicarbazide in dilute acetic acid to form a milky solution which deposits fine white crystals after standing several hours. These crystals were only slightly soluble in water, but moderately soluble in alcohol. Recrystallized several times from alcohol and water they melted at 161–162°. Their analysis corresponded with that of methylglyoxal-4,4-diphenylsemicarbazone.

$C_{21}H_{19}O_2N_6$.   Calculated.   N 17.14.
                        Found.   " 16.50, 16.49.

TABLE IV.

*Formation of Methylglyoxal from Glyceraldehyde in Presence of Aniline at 25°
As Determined with p-Nitrophenylhydrazine.*

Each solution contained glyceraldehyde 0.125 gm. and acetic acid 1 cc. in a total volume of 31 cc. The number of mols of glyceraldehyde present per mol of aniline for each solution were, Solution A 2.58, Solution B 5.16, Solution C 10.3, and Solution D 20.7. The results as tabulated were obtained by the analysis of 5 cc. portions with p-nitrophenylhydrazine and represent per cent of the theoretical yields.

Time.	Solution A.	Solution B.	Solution C.	Solution D.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*
40	28.3	16.2	8.1	5.0
84	45.4	36.7	19.6	8.7
168	54.7	50.9	33.6	14.9
252	50.8	50.2	36.2	19.1
336	52.2	55.0	41.8	24.7

In view of the slight solubility of methylglyoxal-4,4-diphenylsemicarbazone, 4,4-diphenylsemicarbazide was tested as a reagent for the determination of methylglyoxal formed from glyceraldehyde in the presence of the compounds listed in Table III; substantially the same results were obtained. However, 4,4-diphenylsemicarbazide slowly acts as a catalyzer in the formation of methylglyoxal from glyceraldehyde so that the results were not as definite as those obtained with *m*-nitrobenzhydrazide as the reagent.

Formation of Free Methylglyoxal from Glyceraldehyde and Aniline.—In order to show that methylglyoxal formed from glyceraldehyde by the catalytic action of aniline would accumulate in

solution and not undergo complete rearrangement into lactic acid or other compounds, several experiments were carried out in which small quantites of aniline were added as compared to the amounts of glyceraldehyde present.[2]   Solutions, the composition of which is shown in Table IV, were permitted to stand at 25°.   After definite periods aliquot portions were analyzed for methylglyoxal with p-nitrophenylhydrazine.   These and other similar experiments conducted for longer periods show that the quantity of methylglyoxal formed reaches a maximum which is slightly more than 50 per cent of the theoretical and then slowly falls off.   That the glyceraldehyde in solution had completely disappeared when the maximum yield of methylglyoxal had been obtained was demonstrated by precipitating the methylglyoxal with m-nitrobenzhydrazide, filtering, and adding an excess of aniline to the filtrate.   After standing several days such solutions always failed to deposit a precipitate.   The fate of the glyceraldehyde which was not converted into methylglyoxal has not been determined.   Attempts to isolate pyruvic acid as the p-nitrophenylhydrazone gave negative results.

<div align="center">DISCUSSION.</div>

From the evidence presented in this paper it is clear that glyceraldehyde   and   dihydroxyacetone   undergo   rearrangement   as follows:

$$CH_2OH \cdot CHOH \cdot CH=O \rightarrow CH_3C=O \cdot CH=O + H_2O$$

$$CH_2OH \cdot C=O \cdot CH_2OH \rightarrow CH_3C=O \cdot CH=O + H_2O$$
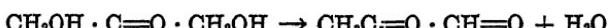
Moreover, it appears that these reactions are not dependent upon the complete interconversion of the trioses, for if in the presence of an amine glyceraldehyde formed dihydroxyacetone, the latter would be incompletely converted into methylglyoxal, while if dihydroxyacetone were converted into glyceraldehyde, this triose would not form methylglyoxal in the absence of a catalyst.   On the other hand, it is possible that the formation of some common

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[2] It is important that the concentration of the amine be rather low as many amines react with methylglyoxal to form slightly soluble ill defined methylglyoxalamine compounds.

enediol modification of the trioses is essential to the rearrangement to methylglyoxal, for example,

$$CH_2OH \cdot CHOH \cdot CH=O \rightarrow CH_2OH \cdot CHOH=CHOH \leftarrow CH_2OH \cdot C=O \cdot CH$$
$$\downarrow$$
$$CH_3C=O \cdot CH=O$$
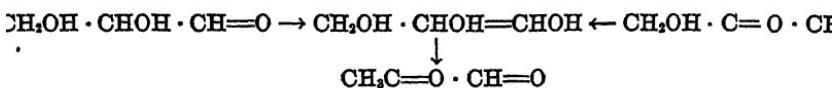
Although we are ignorant of the part played by the catalyst, it is important to note that the reaction takes place under conditions which approximate those of many fermentation media.

URINARY GALACTOSE IN MEN AND WOMEN AFTER THE INGESTION OF GALACTOSE.

BY VICTOR JOHN HARDING AND OLGA MOBERLEY.

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The ingestion of 50 or 100 gm. of glucose is not followed by the appearance of glucose in the urine in at least 50 per cent of normal individuals. Those who show glycosuria come under the heading of renal glycosurics or are exhibiting the phenomenon of glycosuria attributable to the Maclean lag type of blood sugar curve. It is otherwise with galactose. Very few normal individuals would fail to show an amount of urinary sugar demonstrable by one of the many well known qualitative tests after an ingestion of 50 gm. Even the ingestion of 20 or 30 gm. will many times show a qualitatively demonstrable melituria in normal people. Thus compared with glucose, galactose tolerance has been termed low, though the amount in the urine may be small and the utilization of the galactose may be over 90 per cent. The earlier workers in this field gave varying values to the normal tolerance as determined qualitatively by the galactosuria, and used varying criteria for its determination. Rowe (1) summarizes these values in a short table. Briefly they lie around the figure of 30 to 40 gm. As undoubted cases of hepatic cirrhosis had given lower values than the normal, the earlier German workers had attempted to use galactose as a specific liver function test. From this point of view the galactose tolerance test is now recognized to have little or no specific value, but Rowe (2), more recently, in a series of papers has claimed a value in the diagnosis of certain endocrine disorders. As an integral part of his work Rowe recognizes a distinction between adult men and women in their response to galactose ingestion. Normal men show a melituria after 30 gm. ingestion; normal women require 40 gm. before showing a melituria. Rowe's criterion of a melituria is a demonstrable reduction of Benedict's

qualitative sugar reagent. Not only does the normal female differ from the normal male but during her complete sexual life she possesses at least three distinct levels of tolerance. The prepubertal years are characterized by a galactose tolerance of 20 gm., the intermenstrual period of adult maturity by 40 gm., and the post-menopausal may be characterized by 30 gm. Differences from the normal adult level were also noted during the menstrual period and pregnancy. In an investigation covering the excretion of various sugars after their oral ingestion and using sensitive quantitative methods, Folin and Berglund (3) noted the excretion of galactose after an ingestion of as small an amount as 10 gm. They were unable to determine the existence of a renal threshold for galactose. Harding and van Nostrand (4), using slightly different analytical methods, confirmed their results.

The determination of a galactose tolerance by a qualitative urinary examination depends on the arbitrary selection of some concentration of galactose which shall be designated as a positive result, and which shall be called negative if the concentration is below that particular figure. The results lose the value of precise figures, for the concentration of galactose in urine, which shows a positive test with Benedict's qualitative sugar reagent, is variable, and depends on the concentration of other salts and substances. It is evident that significance can only be attached to results obtained in this way if the differences between different groups are well outside the variation of the individuals comprising such groups. Or that if there are exceptions, such exceptions form only a small percentage of the total.

Harding and van Nostrand recently published figures on the elimination of galactose in five normal men and two women after the ingestion of 20, 30, and 50 gm. of galactose. They made also observations on the blood and urinary sugar, after the ingestion of 50 gm. of galactose on nine other normal males. The quantitative results on the men showed great variation. The results on the two women placed one with a very low tolerance, and the other with a very high tolerance towards galactose, and led to the supposition that the examination of a series of women in this way would show as big a variation as had been found in males. It made it unlikely that a sharp sex differentiation could be made in this respect. The present paper shows the effects of the ingestion

of various amounts of galactose on its urinary excretion on twenty normal men and twenty-two normal women, including the previous observations.

Subjects and Methods.

The male subjects were either laboratory workers in our own department, or medical students in one of the classes conducted by the department where we had an opportunity of personal observation. The female subjects were medical students, graduates, or nurses. All were free from any recognizable clinical disorder, all urines on examination were free from albumin, and their weights taken in conjunction with their age and height were within normal variation, with two doubtful exceptions among the women. Care was taken to carry out the observations on the women during the intermenstrual period. Succeeding observations on the same individual were made at not less than weekly intervals in order to insure absence of influence of the first upon the second, or succeeding tests. The schedule of testing followed that given by Harding and van Nostrand. A fasting urine specimen collected 1 hour after the ingestion of 200 cc. of water or weak tea without sugar formed the zero or control specimen. The required amount of galactose in 200 cc. of water followed by a further 200 cc. of water was then taken and the urine collections made every hour for the succeeding 3 hours. Tests by the Benedict qualitative sugar reagent were applied to each specimen of urine. The first observation made on any individual was with 30 gm. of galactose. Depending on whether the Benedict test was positive or negative in any of the urine specimens, a second observation was made with either 20 or 40 gm. In this manner we were able to determine the amount of galactose necessary to produce a positive urinary Benedict test to within 10 gm. Following the definition of Rowe this would be called the galactose tolerance of the individual. The method differs from that of Rowe only by making hourly instead of 2 hourly collections of urine, and in keeping a constant water intake. Each urine specimen was analyzed for fermentable and non-fermentable sugar by the methods given by Harding and van Nostrand. The galactose used was Schuchardt's "ordinary." In view of Rowe's emphasis on the influence of impurities on the final result by the Benedict test, we must again

repeat that no difference, beyond that to be expected as a result of normal individual variation, was observed to follow the use of galactose recrystallized twice from aqueous alcohol, and galactose as obtained from the manufacturer. Recrystallization raised slightly the specific rotation of the sugar, but the galactose tolerance of the individual was unaffected.

TABLE I.

Showing the Excretion of Galactose in Normal Men after the Ingestion of Varying Amounts of Galactose.

Subject.	Age.	Height.		Weight.	Output of galactose after ingestion of:				
					10 gm.	20 gm.	30 gm.	40 gm.	50 gm.
	yrs.	ft.	in.	lbs.	mg.	mg.	mg.	mg.	mg.
V. E. B............	26	5	11	168			90	376	164
R. H.............	27	5	10	156			253	716	
W. J. E...........	27	5	8	156			71		
W. B. S...........	22	6	0	187			131	219	
F. G. C..........	24	6	0	190		116	226		
R. W. C..........	26	5	11½	158	35	428	348		
M. A. N..........	25	5	10	147	27	184	390		
F. O. D..........	22	5	7	142	111	614	1146		
H. K.............	23	5	7	166		83	348		
T. W. D..........	22	5	11	138		49	638		
R. G. C. K.......	23	5	10	126			107	157	320
H. S.............	22	5	9	146		94	254		
D. L. A........ ..	24	6	1	165		391	836		
D. L. S...........	25	6	0½	177			123	216	
R. P. F...........	22	5	10½	150			67	115	
T. F. N...........	28	5	5½	135			27	132	218
C. C. L...........	25	5	10½	136			77	221	
F. H. v. N.......	32	5	8	170			55	307	
V. J. H...........	43	5	9	144			145	272	
C. E. D..........	33	5	8½	154	?		430	826	

Average, after 30 gm. ingestion................... 339 mg. galactose.

RESULTS AND DISCUSSION.

Excretion of Galactose.—The calculation of the amount of galactose excreted is that given by Harding and van Nostrand. The amounts after the ingestion of the varying doses of galactose are shown in Tables I and II. An inspection of the tables will render

it evident that there is a great deal of variation both in men and women, thus supporting the facts previously observed. Moreover, with a larger number of subjects, anomalous results begin to make their appearance. Thus R. W. C. (men) excretes less on 30 gm. ingestion than on 20 gm., I. R. (women) excretes less on 40 gm.

TABLE II.

Showing the Excretion of Galactose in Normal Women after the Ingestion of Varying Amounts of Galactose.

Subject.	Age.	Height.		Weight.	Output of galactose after ingestion of:				
					10 gm.	20 gm.	30 gm.	40 gm.	50 gm.
	yrs.	*ft.*	*in.*	*lbs.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
C. F............	21	5	8	132			573—	989	
H. McN.........	24	5	5	129			214—	430	
P. M. B.........	29	5	2	135			135—	472	756
G. W............	22	5	0·	124		307	1201—	1295+	
M. S............	35	5	6	129			356—	790	·
P. S.............	29	5	7½	136½			562—	1690	
R. M. P.........	33	5	5	136¼		317	1048—		
E. C............	40	5	6	181		126	566—		
R. J.............	27	5	2½	115		132	514—		
D. M............	32	5	5½	140			163—	171	
M. E. B.........	26	5	9	130			607—	995	
I. R.............	26	5	4	144			961—	938	
E. McK.........	44	5	4½	114		92	179—		
F. P. S..........	29	5	1½	114			98—	809	
I. McC..........	26	4	8	87			230—	235	
M. R............	22	5	4	135			160—	393	
H. C............	26	5	3½	128			261—	1409	
M. H. G........	26	5	7½	138		16	86—		
E. G............	24	5	4	135			182—	163	619
C. A. S.........	24	5	7	144			168—	191	470
Miss A..........	26	5	9	151			206—		394
Miss B..........	25	5	1½	98		566	1639—		

Average, after 30 gm. ingestion................... 450 mg. galactose.

than on 30 gm., and I. McC. (women) excretes only 5 mg. more galactose on 40 gm. than on 30 gm. In different subjects, both male and female, the transition of 10 gm. of galactose sometimes produces a large and sometimes a very small difference in the excretion. These differences are perhaps to be correlated with

differing food intake in the interval between the two tests. We may examine this point later. The different excretions of the various subjects are so great that any average has very little value. The average value for women on 30 gm. ingestion of galactose is 450 mg. For men the similar average is 339 mg. The difference of 111 mg. would appear to show that women have a lower tolerance for galactose than men—a conclusion exactly opposite to that reached by Rowe. When one remembers, however, that the ingestion of galactose is in gm. and the excretion is reckoned in mg., and that from the standpoint of utilization, the figures show a 98.50 per cent utilization by women, and a 98.87 per cent utilization by men, any idea of drawing a sex distinction must be abandoned.

TABLE III.

Summary of Results of Rowe and the Authors Showing the Behavior of Urine towards Benedict's Sugar Reagent after Ingestion of Galactose.

Galactose ingestion required to produce galactosuria demonstrable by Benedict's test.	No. of subjects showing positive Benedict's test.					
	Men.		Women.		Total.	
	Rowe.	Authors.	Rowe.	Authors.	Men.	Women.
10 gm. negative; 20 gm. positive..	3	4	0	2	7	2
20 " " 30 " " ..	10	8	5	5	18	10
30 " " 40 " " ..	5	5	6	11	10	21
40 " " 50 " " ..	0	3	5	4	3	9

Reckoned statistically the probable error of our experiments on women is ±287 and on men is ±193. As the probable error is nearly three times the difference of the means in women and nearly twice the difference in men, it becomes unwise to draw any conclusion.

Reaction with Benedict's Reagent.—Rowe's conclusions on a sex difference in the behavior towards galactose were based, however, not on a quantitative estimation of the galactose excretion, but on a qualitative test. Moreover, as we have pointed out there is a slight difference between the technique of Rowe and ourselves. What is the response of our subjects towards Benedict's qualitative sugar reagent applied to hourly urine specimens after the ingestion of the galactose? As a positive response after 30 gm. ingestion

was followed by a second test with 20 gm. and a negative response after 30 gm. ingestion by one of 40 gm., the general results can be inferred from Tables I and II. Even a general inspection of the tables will show that the greater proportion of our women required the ingestion of 40 gm. or over to produce a positive Benedict sugar test, while with our male subjects over 50 per cent showed a positive result with 30 gm. and four of the subjects were still positive with 20 gm. From this standpoint our experiments agree with those of Rowe, despite the slight variation in technique. Table III shows the results of ourselves and Rowe. According to Table III there seems a distinct tendency for men to show a positive urinary Benedict test with an ingestion of less galactose than women. The number of reported observations, however, is small, and does not in our opinion warrant the fixing of the galactose tolerance as determined in this way at any one particular figure. If we accept the figure for galactose tolerance in men as 20 to 30 gm. barely 50 per cent fall in the group. Exactly 50 per cent of women show a galactose tolerance of 30 to 40 gm.—Rowe's normal value. To establish Rowe's normal figures requires a much larger number of cases than those at present published, with a much narrower range of deviation.

Despite the lack of a sex difference in galactose tolerance as shown by quantitative examination of the galactose excretion, the tendency for a difference in reaction of the urine of men and women towards Benedict's qualitative sugar reagent under the same circumstances invites investigation. This reaction is brought about by the reducing power of the fasting non-fermentable carbohydrate substances normally occurring in urine and the excreted galactose. The amounts of urea, ammonium salts, and creatinine would also influence the sensitivity of the test. Of this last factor in our particular series of experiments we have no knowledge, nor do we know any mention in the literature of a sex difference in the fasting excretion of urea and ammonium salts. Women, however, are known to excrete a smaller amount of creatinine and often excrete creatine. Our observations supply a certain amount of information on the other factors affecting the reaction towards Benedict's reagent.

Output of Fasting Non-Fermentable Reducing Substances.—In the course of this and the previous investigation of Harding and van

Nostrand we have collected observations on a number of men and women on the output of urinary non-fermentable reducing substances in the fasting subject between the hours of 9.00 to 10.00 a.m. The data are collected in Table IV. Twenty men, in 55

TABLE IV.

Hourly Output of Urinary Non-Fermentable Reducing Substances in Fasting Subjects.

	Men.		Women.	
Subject.	Non-fermentable sugar as galactose.		Subject.	Non-fermentable sugar as galactose.
	mg.			*mg.*
D. L. S.........	24, 29		C. F...........	115,* 32
R. P............	24, 36		H. McN........	23, 13
J. L. A.........	13, 11		P. M. B........	16, 16, 20
H. S............	18, 22, 21		C. A. S.........	25, 18, 16
W. J. E.........	14		G. W...........	21, 12, 19
W. B. S.........	26, 15		R. M. P........	14, 26
V. E. B.........	30, 22, 34		M. S...........	27, 22
R. H...........	18, 7		E. C...........	27, 26
F. G. C.........	27, 27		I. McC.........	15, 16
R. W. C........	19, 41,* 23		D. P. S........	34, 30
M. A. N........	32, 25		M. E. B........	17, 18
F. O. D.........	26, 12, 11		D. M...........	22, 30
H. K...........	25, 17		R. J...........	14, 19
T. W. D........	29, 19		E. G...........	23, 28, 22
R. G. C. K.....	23, 21, 21		I. R...........	20, 23
C. L. D.........	18, 20, 21		H. C...........	17, 19
C. C. L.........	18, 18, 19, 25, 30, 31		M. G. H......	34, 34
V. J. H.........	33, 35, 31, 33, 21, 29		E. M. K......	32, 30
T. H. N........	30, 22		F. P. S........	23, 26
F. H. v. N......	64,* 32, 24, 21		M. P...........	17, 19
			Miss B.........	20, 21, 34
			Miss A........	15, 13, 17, 21

* Values not considered in determining the average discussed in the text.

observations, show an average of 23.6 mg.; twenty-two women, in 51 observations, show an average of 21.3 mg. It is evident that no difference is observable between men and women in this respect. The general hourly average of men and women is 22.5 mg. of non-fermentable carbohydrate-reducing substance expressed as galac-

tose or 23.4 mg. as glucose.   If this rate is maintained throughout the 24 hours it would represent an output of 561 mg. as glucose. The two subjects of Benedict, Osterberg, and Neuwirth (5) showed an hourly excretion of total sugar of approximately 30 and 20 mg. per hour.   Those of Folin and Berglund showed similar figures.

Three anomalous values will be seen in Table IV and are noted by an asterisk.   These were excluded in determining the average. They may represent extreme variations but as the subjects showed more normal values at other times, it is more probable that they
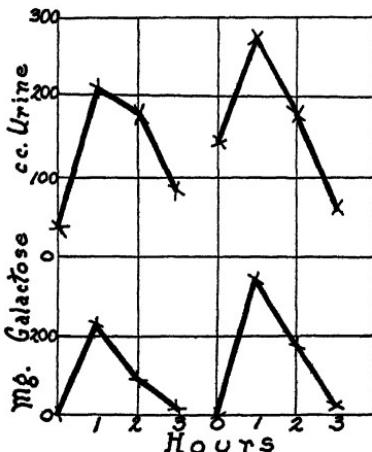
Fig. 1. Showing the hourly output of urine and galactose after 30 gm. ingestion.

represent errors in analysis or in the collection of the specimen. Duplicate determinations, however, on the same urines gave concordant results.

Output of Water.—As the output of fasting non-fermentable carbohydrate material shows no sex difference and the total output of galactose is, if anything, a little higher for women than for men when on the same intake, any difference in the reaction of the urine specimens towards Benedict's reagent would appear to depend on the concentration of these substances.   Fig. 1 shows the hourly output of urine of men and women under the test conditions and the hourly output of galactose; both observations after

the ingestion of 30 gm. of galactose. The galactose excretion shows a peak in the 1st hour, thus confirming the general results of the previous paper and is throughout slightly higher for women than men. The output of urine in the 0 and 1st hours is, however, considerably higher for women than men. There would appear to be a sharper response to the diuretic effect of the water. This would lower the concentration of the galactose at the peak of its excretion, and demand a higher intake by women in order to give sufficient concentration to show a positive Benedict sugar test. If the explanation of the difference between the qualitative and quantitative response to galactose tolerance tests lies in the concentration, rather than the amount, of the excreted reducing substances, then the excretion of the galactose after a 30 gm. dose must hover on the threshold of the sensitivity of the Benedict test. The average concentrations of galactose at the peak of excretion are 99 mg. and 94 mg. per 100 cc. of urine for men and women respectively. This difference is slight, individual variations again are great, and it seems more than possible that the examination of a larger number of cases will reveal no detectable difference between men and women. In the meantime, we prefer to ascribe such differences as have been found to differences in concentration rather than to any inherent sex ability of the female, or disability of the male.

SUMMARY.

The excretion of galactose after the ingestion of varying amounts has been studied in a series of men and women. Great variation is found in both sexes.

On the average, women excrete more galactose than men on the same intake. The variation is, however, too great to allow the conclusion that women have a smaller galactose tolerance than men.

If the Benedict qualitative sugar reagent is used as the criterion of sugar excretion, instead of a quantitative determination, women appear to have a greater tolerance than men. This latter conclusion agrees with that of Rowe. The explanation of the discrepancy may lie in a difference in response to the diuretic action of water rather than in an inherent sex difference towards the metabolism of galactose, but again, individual variation is great, and no definite conclusion can be substantiated.

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THE METABOLISM OF TRICAPRYLIN AND TRILAURIN.*

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Early work by Munk (1), Leick and Winkler (2), Lebedeff (3), Lummert (4), Rosenfeld (5), Henriques and Hansen (6), and others, on the effect of ingestion of foreign fats upon the depot fat of animals, showed a striking change in the iodine number of the depot fat, corresponding to the character of the fat in the food. When mutton fat was fed the iodine number went down; when linseed oil was fed, it went up. The unsaturated acids of the depot fat are evidently influenced by those in the food. Lummert (4), Henriques and Hansen (6), König (7), Gibbs and Agcaoli (8), and others have found that feeding butter fat or coconut oil results in a lowering of the iodine number of the depot fat. This pointed to a deposition of some or all of the lower saturated fatty acids of these oils, at the expense of unsaturated acids. Experiments by Leube (9) and Zuntz (10) in feeding butter fat, and by Lebedeff (11) in feeding tributyrin, show very little, if any, increase in volatile fatty acids in the depot fat. Later experiments by Eckstein (12–14) and by Davis (15) in feeding the butyric and caproic radicals resulted in no change in the saponification number of the depot fat, but a significant decrease in the iodine number. While there is no clear evidence that these two lower acids are deposited, their ingestion in large amounts alters the character of the body fat. An experiment by Eckstein (12) in feeding myristic acid (14 carbon atoms) to white rats, resulted in a body fat containing 17.6 per cent myristic acid.

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These experiments have left the fate of the lauric, capric, and caprylic radicals still in doubt and the present paper is a report of the effect of feeding lauric and caprylic acids to white rats.

EXPERIMENTAL.

Caprylic and lauric acids were isolated from coconut oil by saponificaticn and fractional distillation, *in vacuo*, of the free acids. The triglycerides were prepared by the method of Scheij (16), a slow stream of carbon dioxide instead of air being used. Male albino rats which had almost gained adult size were used in preference to young animals. They were fasted until they lost

TABLE I.

Composition of Diets Administered.

	Diet A.	Diet B.	Diet C.
	per cent	*per cent*	*per cent*
Corn-starch................................	72	50	50
Cane-sugar................................	8	5	5
Salt mixture................................	5	5	5
Ether-extracted casein......................	15	15	15
Tricaprylin................................		25	
Trilaurin................................			25

The salt mixture was that of Osborne and Mendel (17). The cane-sugar was added to make the mixture more palatable. The casein was extracted with ether for 3 days, the ether being changed each day.

about 25 per cent of their weight and were then fed on the special diets until they had practically regained the lost weight. Control animals were given a diet in which the fat was replaced by starch. The diets are given in Table I.

In addition each rat was given separately each day a vitamin mixture composed of 0.1 gm. of dried spinach, 0.4 gm. of dried yeast obtained from the Northwestern Yeast Company, and 0.01 gm. of oscodal (unsaponifiable fraction of cod liver oil).

The rats ate well and within 3 to 4 weeks regained their original weight, or very near to it. They were then killed with chloroform, the skin was removed, and the abdominal fat dissected out. The skins with the subcutaneous fat and the abdominal fatty tissue were extracted with a mixture of 3 parts of 95 per cent alcohol

and 1 part of ether.   When extraction was complete, the solvents were distilled off under reduced pressure, the residue taken up with anhydrous ether, and filtered.   The ether was evaporated at

TABLE II.

Data for Rats on Various Diets.

Rat No.	Initial weight.	Weight after fasting.	Loss in weight.	Final weight.	Days fed.	Weight of fat.	Saponifi- cation No.	Iodine No.
				Control rats on Diet A.				
	gm.	*gm.*	*per cent*	*gm.*		*gm.*		
101	279	218	22	269	24		196	69
102	250	199	20	251	19		200	71
103	278	216	22	273	21		195	72
104	309	240	22	293	21		200	60
105	256	196	23	265	19		202	72
106	299	231	23	287	21		199	70
Average for Diet A................................							199±1	70.5±0.4
				Rats on Diet B (tricaprylin).				
113	262	201	23	257	23	22	199	63
114	289	223	23	280	24	22	203	65
115	223	173	21	223	20	12	201	63
116	235	183	22	245	20	16	203	63
117	278	220	21	274	20	13	203	66
Average for Diet B................................							202±1	64.0±0.4
				Rats on Diet C (trilaurin).				
107	199	156	22	200	28	13	220	41
108	164	205	22	259	23	21	215	50
109	311	232	25	303	28	11	219	45
110	274	213	22	266	24	23	218	45
111	272	209	23	268	23	28	214	52
112	282	208	26	257	29	9	222	36
Average for Diet C................................							218±1	44.8±1.6

room temperature and the fat finally dried in a vacuum desiccator for a week.   Saponification and iodine numbers were determined in each case.   The results are given in Table II.

Further analysis of the fat was made as follows:

Fat from Rats Fed on Diet B.—The increase in the saponification number was hardly enough to be significant, but the decrease in the iodine number clearly indicated a change in the fat. In order to detect any volatile fatty acids, the Polenske and Reichert-Meissl numbers were determined (Table III).

If the fatty acid represented by both the Reichert-Meissl and Polenske numbers were caprylic acid, it would give a maximum of only 1.5 per cent of the acid in the fat. An effort was made to isolate caprylic acid by converting 40 gm. of the fat into the methyl esters and distilling *in vacuo*. Several cc. of an ester

TABLE III.

Analysis of Fat of Rats on Diet B.

Rat No.	Reichert-Meissl No.	Polenske No.
113, 114	4.5	1.5
115, 116, 117	3.8	1.3
Fat-free diet (13).	0.62	0.59

TABLE IV.

Analysis of Fat of Rats on Diet C.

Fat from Diet C.	Neutralization No.	Iodine No.	Per cent of total acids.
Saturated acids..........................	239	10	63
Unsaturated "	198	89	37

boiling at 83–84° under 15 mm. pressure were obtained. This was saponified and the caprylic acid was isolated as the magnesium salt. The resulting acid had a neutralization number of 362 (theoretical for caprylic acid, 389). About 0.15 gm. of material was isolated.

Fat from Rats Fed on Diet C.—The results in Table II indicated clearly that lauric acid had been deposited in the depot fat. To determine the extent of its deposition, the saturated acids were separated from the unsaturated acids by the lead salt-ether method. Neutralization and iodine numbers were determined (Table IV).

Assuming that no stearic acid was present, these results gave 43.2 per cent of lauric acid in the saturated acids.   The amount of lauric acid was checked by preparation of the methyl esters and repeated distillation *in vacuo*.   Saponification numbers were made on these fractions and the amount of lauric acid was calculated as 40 per cent.   There was little or no stearic acid.   From the data on the methyl esters the composition of the fat of the rats fed on trilaurin is given in Table V.

DISCUSSION.

When trilaurin was fed, 25 per cent of the fatty acids of the depot fat was lauric acid.   Eckstein (12) found only 17.6 per cent myristic acid but he was working with growing animals, fed over a period of 8 weeks.   His rats did not eat the food well and the slow deposition of reserve fat would give more chance for modification

TABLE V.

Distribution of Fatty Acids in Fat of Rats on Diet C.

	per cent
Lauric acid	25.2
Palmitic acid	31.5
Oleic acid	43.3

than in the present case, where the rats ate well and deposited large stores of fat in 3 to 4 weeks.   It seems probable that under conditions as favorable for deposit as those of the present experiment, as much or more myristic acid would be found than lauric acid.

The neutralization number of the caprylic acid does not check very well with the theoretical, but such a small amount was available that the experimental error was large, and there may have been impurities in it, which would tend to make it too low.   However, it is much nearer the value for caprylic than for capric acid, and it seems certain that feeding tricaprylin resulted in the deposition of traces of caprylic acid.   This would explain the doubtful effect upon the Polenske number which has been reported by Gibbs and Agcaoli (8), Lührig (18), and Siegfeld (19) after feeding coconut oil.   There is enough caprylic acid deposited to alter the Polenske number; but since the experimental error in determining

the Polenske number is large, the change does not appear to be significant.

The general effect of feeding tricaprylin agrees with that reported by Eckstein for the butyric and caproic radicals. The saponification number of the fat is not altered to any degree but there is a distinct lowering of the iodine number. If the caprylic acid had been responsible for the lowering of the iodine number (by replacing oleic acid), then the saponification number must have been raised. The feeding of the lower fatty acids results in an increase in the proportion of the ordinary long chain saturated acids. This experiment furnishes no evidence as to where or how this change takes place.

SUMMARY.

Feeding trilaurin to rats produces a depot fat containing as much as 25 per cent lauric acid.

Feeding tricaprylin results in the deposit of caprylic acid, but only in traces. As in the case of feeding butyric and caproic acids, there is a distinct lowering of the iodine number of the body fat but there is little change in saponification number. The present experiment throws no light upon how this increase in long chain fatty acids is brought about.

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THE RECOVERY PROCESS AFTER EXERCISE IN THE MAMMAL.

I. GLYCOGEN RESYNTHESIS IN THE FASTED RAT.

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The Hill-Meyerhof theory of the recovery process occurring in the muscles after exercise postulates that four-fifths of the lactic acid formed is reconverted into muscle glycogen, while the remainder is oxidized. Since Meyerhof has shown that this cycle occurs in the isolated frog muscle, then the muscles themselves must be able to effect this transformation.

The exact pathway by which lactic acid is converted into glycogen is not definitely known.

In mammalian skeletal muscle the evidence is by no means so clear that the muscles themselves can transform lactic acid into glycogen without the intervention of the liver. Elias and Schubert (1) and Janssen and Jost (2) have demonstrated that while injected lactates are rapidly removed from the blood, there is no immediate deposition of muscle glycogen and no increase in oxygen consumption by the muscles.

The resynthesis of muscle glycogen after exercise in the fasting mammal has been studied in a few instances only. Long (3) found in cats under amytal anesthesia that 3 hours after exercise there was little or no restoration of muscle glycogen, but in non-anesthetized animals glycogen deposition occurred more rapidly. McKay (4) found in decapitate preparations that the glycogen content of exercised muscles did not increase after 3 hours recovery. Hinsey and Davenport (5) state that they have confirmed Long's findings on animals under amytal anesthesia.

The mammalian liver can transform *d*-lactates fed by mouth into liver glycogen with great ease. This has been demonstrated

by Cori and Cori (6). Following the injection of epinephrine muscle glycogen is partly broken down into lactic acid, and this lactic acid is converted into glycogen in the liver (Cori and Cori (7)). After hepatectomy muscle glycogen decreases and lactic acid accumulates in the blood in large amounts (Bollman, Mann, and Magath (8); Kilborn, Soskin, and Thomas (9)). From these and other observations Cori and Cori (6) and Himwich, Koskoff, and Nahun (10) have put forward the view that a cycle occurs in the mammal in which muscle glycogen is converted to lactic acid, the lactic acid passes to the liver, and is there transformed into glycogen. The liver glycogen can then replenish the muscle glycogen through conversion into glucose.

During muscular exercise in the mammal large amounts of lactic acid pass into the blood stream. In view of this it became of interest to investigate the part played by the above cycle in recovery after exercise. Most of the evidence would indicate that once lactic acid has left the muscles the only pathway by which it may return is by conversion in the liver into glycogen. The liver glycogen can then return carbohydrate to the muscles in the form of blood glucose. If this is so then a certain portion of the lactic acid produced during exercise becomes subject to all the influences which normally control glucose deposition as glycogen and oxidation in the muscles at rest. It would indeed be interesting if the recovery process after exercise is dependent as much upon the liver and pancreas as upon the muscles themselves.[1]

In the present paper we shall confine ourselves to the findings on the time relationships of glycogen deposition and lactic acid removal after exercise in the mammal.

Methods.

Animals.—Rats were used in all the experiments. They were derived from descendants originally obtained from the Wistar Institute. They were kept upon a stock diet of dried milk, whole wheat flour, and a salt mixture. In addition, green stuffs and chicken scraps were fed once or twice a week.

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[1] Since this was written Debois (11) has shown: (1) that the integrity of the left vagus nerve is necessary for muscle glycogen resynthesis after exercise and (2) that there is no deposition of muscle glycogen in the depancreatized animal after exercise, but this does occur when insulin is given.

The weight of the rats used varied considerably owing to the large number of experiments done and the impossibility of obtaining sufficient animals of approximately equal weight. Control values over the whole range of weights used did not show a greater variation than did those animals of approximately equal weight. Both males and virgin females were used indiscriminately. They were all fasted for 24 hours before an experiment.

Exercise.—Since the success of the investigation depends upon the comparison of a large number of animals under different conditions it was very necessary that some method of exercise be used that would give fairly equal reductions of whole body glycogen from animal to animal. We know from the work of Cori and Cori (12) that the liver and body content of glycogen of the resting 24 hour fasted rat is remarkably constant.

Any method which depends upon voluntary exercise on the part of the rats themselves (such as a revolving cage), while useful for individual observations, is of no use when we wish to observe the glycogen content at various times after exercise, since each rat will reduce its stores of body glycogen by different amounts depending upon the amount of work performed and its inclination for this work.

Finally a method of artificial exercise was adopted which although imperfect in some ways does yield fairly constant reductions in body glycogen, so that observations on different animals become comparable.

The rat was fastened on its abdomen to a small board by its four limbs. A small drop of novocaine (0.5 per cent) was injected under the skin of the back in the upper dorsal and lower lumbar regions. The skin over the areas was then incised and a small brass electrode 1 cm. in diameter was sewn in. The terminals of the electrodes were connected to the secondary leads of a Palmer induction coil in the primary circuit of which was a Lewis rotary contact so arranged as to deliver the break shock only. The current was supplied by two dry cells (3 volts).

By this method not only could the speed of exercise be regulated but also the intensity of the contractions. The latter was done by altering the distance of the secondary coil from the primary.

The speeds of exercise studied were 180, 60, and 20 a minute, the secondary coil in all cases being 7 cm. from the primary and

the exercise continued for 10 minutes. It is important once the apparatus is set up that the same induction coil is used throughout and also that a close watch be kept on the condition of the dry cells.

The effects of the exercise were of-some interest. The contractions affected practically all the muscles of the body. At the rate of 20 a minute they did not diminish greatly in strength during the period of exercise. At 60 a minute there was some decrease and this was very marked at the highest speed used— 180 a minute. The respiration and heart rate increased greatly but no records were obtained. No observations were made on the blood pressure but the condition of the circulation as far as could be judged appeared to be good.

When the animals were released after the exercise, they were rather cramped for a few minutes but in most cases soon regained the use of their limbs and walked around normally. When left to themselves, however, they usually curled up and apparently went to sleep, displaying a marked disinclination for further exercise.

The changes in body temperature during and after these artificial exercises were carefully followed in the majority of the animals. All temperatures were taken with a small rectal thermometer, which was left in place for 2 minutes before a reading was taken. The average body temperature of 100 of our rats taken just before the exercise was $37.4° \pm 0.6°$. During the exercises at the lower speeds it sometimes rose $0.5°$ or fell a similar amount. At the highest rate of speed in the majority of animals similar variations occurred but in some animals a much more marked fall was observed. All animals in which the body temperature fell more than $1.5°$ either during exercise or recovery were rejected from our series.

If the rate of stimulation was greater than 180 a minute or if the distance between the primary and secondary coil was lessened, then marked falls of body temperature occurred during exercise and in addition the fall continued after the exercise was over. In some instances temperatures as low as $30°$ were recorded. The explanation of this is, either that the animals were in a condition of shock, which was rather borne out by the congested appearance of the viscera at autopsy, or else the glycogen stores of the body were so greatly depleted that the body temperature fell from

lack of combustible carbohydrates in the muscles to support it. Obviously a complication of this kind renders the experiment useless and illustrates the necessity for a close watch on the body temperature during such experiments.

Killing and Freezing.—At the end of the experiment the rats were killed with coal gas and were bled by cutting the throat. The abdomen was then split open and the liver removed and frozen with carbon dioxide from a tank. The remainder of the viscera were then removed and the carcass skinned.

The skinned, eviscerated carcass which consists chiefly of the skeletal muscles, along with the thoracic viscera and the skeleton, was then frozen.

The freezing of both the carcass and liver was done in stout wooden boxes of a suitable size, which were provided with a lead lid. Short brass tubes attached to the nozzles of the carbon dioxide tanks projected into the boxes and several orifices were provided for the escape of the gas. Before use the box for the liver was filled with solid carbon dioxide and a certain amount was also placed in the box for the carcass. The tissues were then placed in them and the gas blown through. By this method very rapid and even freezing of both the liver and carcass could be obtained with a little practice. We found it best to use the syphon variety of carbon dioxide tanks as the gas was much dryer and did not choke the orifices with ice. The time for these operations need not be more than a minute in practiced hands.

Chemical Analysis.

Glycogen.—Glycogen was estimated by Pflüger's method. The sugar formed by its hydrolysis was estimated by Somogyi's (13) modification of the Shaffer-Hartman method.

Lactic Acid in the Whole Body.

The rat was skinned and eviscerated, and the skin and viscera chopped up at once in ice-cold 5 per cent trichloroacetic acid. In the meantime the carcass was frozen as before and was then cut into small pieces beneath the chilled trichloroacetic acid solution. The mixture was allowed to remain in the freezing bath for 2 hours and then stood at room temperature overnight. The next day it was made up to known volume and then filtered

Portions of the filtrate were treated with copper and lime to remove interfering substances and the lactic acid estimated by Clausen's method.

Blood lactic acid was estimated by Clausen's method and blood sugar by the Folin-Wu method.

Results.

1. Controls.

The average figures for the glycogen content of the carcass of thirty control rats were 200 mg. ± 36 mg. per 100 gm. of rat.   The weights of the rats cover a fairly wide range, from 119 to 235 gm., but the percentage glycogen content seems to be independent of this.

The average glycogen content of the liver is 0.081 ± 0.04 gm. per 100 gm. of liver.   These figures are subject to a much wider variation than those of the carcass and also are lower than those reported by Cori and Cori (6, 12).   The reason for this is perhaps the different manner in which the rats were killed.   We used coal gas while Cori and Cori stunned the rats.   The longer period of asphyxia probably depleted the liver glycogen .to a greater extent.   We had hoped to reduce the asphyxial struggle by the use of coal gas, but the figures for the blood lactic acid are not any lower than those previously reported (6).

The blood lactic acid averaged 47 mg. per 100 cc. and the blood sugar 104 mg. per 100 cc.

2. Relation between Speed of Exercise and Reduction in Carcass Glycogen.

Table I shows the relation between the rate at which the animal was exercised and the ensuing reduction in carcass glycogen. Even at a rate of 20 a minute for 10 minutes there is an average reduction of 48.5 per cent in glycogen, while increasing the speed 9 times produces an average diminution of 71 per cent.   It would appear that beyond a rate of about 60 a minute no very marked increase in glycogen breakdown can be brought about.   This result bears out those of previous workers who have shown that exercise alone will not entirely deplete isolated muscles of their glycogen store (14).   Meyerhof (15) found in isolated frog muscle

that the maximum amount of lactic acid that could be liberated on stimulation was only about half the amount that could be liberated by mincing the muscle in a buffered phosphate solution.

For the mammal as in the frog it would appear that all the muscle glycogen cannot be converted into lactic acid during exercise. Some other factor or factors come into play which cause it to cease when some 70 per cent of the glycogen has been transformed into lactic acid. In the intact animal failure of the circulation as well as local conditions in the muscles themselves must play a considerable part.

3. Glycogen Resynthesis and Lactic Acid Removal after Exercise.

The length of recovery after exercise was from 1 hour to 24 hours. We had thought that the recovery of muscle glycogen

TABLE I.

Relation between Speed of Exercise and Reduction of Carcass Glycogen.
Average figures.

Speed per min.	Reduction in carcass glycogen.
	per cent
20	48.5
60	63.5
180	71.0

in the intact non-anesthetized mammal would have followed closely the same time relationships as does the removal of excess lactic acid from the blood after exercise. Hill, Long, and Lupton (16) found in man that 90 minutes after the most severe exercise the blood lactic acid and oxygen consumption were nearly at their initial level before exercise. We had surmised therefore that after this time the restoration of muscle glycogen would also be nearly complete. The results in Table II show that for the three rates of exercise studied in the rat this is not so. For all three rates of exercise studied there is a decrease in carcass glycogen after exercise, the degree of which depends upon the rate of exercise. At the lowest rate (20 a minute) the carcass glycogen increases to about 80 per cent of the preexercise level after 2 hours recovery. When the exercise is at the rate of 60 a minute

TABLE II.

Glycogen Content of Carcass and Liver of Rats after Varying Rates of Stimulation and Different Times of Recovery. Average figures.

	No. of rats.	Weight.	$\frac{\text{Carcass weight}}{\text{Body weight}} \times 100.$	Carcass glycogen.	$\frac{\text{Liver weight}}{\text{Body weight}} \times 100.$	Liver glycogen.	Blood sugar.	Blood lactic acid.
		gm.		mg. 100 gm. carcass		mg. per 100 gm. liver	mg. per 100 cc.	mg. per 100 cc.
Controls........................	30	147±28	63.6	200±36	2.83	81	104	47
20 per min. for 10 min.; no recovery....	5	174±24	65.0	103±25	2.38	66	72	151
20 " " 10 " " 1 hr. "	5	181±46	63.3	144±45	2.46	137	95	45
20 " " 10 " " 2 hrs. "	5	203±37	61.5	158±54	2.61	108	105	45
60 per min. for 10 min.; no recovery....	5	182±22	65.7	73±10	2.63	31	73	200
60 " " 10 " " 1 hr. "	5	191±44	62.8	82±17	2.37	45	104	42
60 " " 10 " " 2 hrs. "	15	175±37	62.6	104±27	2.46	55	94	39
60 " " 10 " " 5 " "	10	167±23	61.6	120±21	2.87	31	92	36
60 " " 10 " " 24 " "	5	164±19	65.5	172±41	2.58	61	96	48
180 per min. for 10 min.; no recovery....	10	138±19	65.1	58±15	3.20	122	73	200
180 " " 10 " " 1 hr. "	10	142±15	64.6	66±19	3.07	34	104	42
180 " " 10 " " 2 hrs. "	10	142±17	64.3	88±8	3.05	45	94	39
180 " " 10 " " 5 " "	2	155	64.0	146	2.95	31	92	36
180 " " 10 " " 24 " "	2	150	65.7	171	2.98	61	96	48

there is a slow steady increase in carcass glycogen but it is not until some 15 hours after exercise that the carcass glycogen is at its initial level again.   At a rate of 180 a minute similar results were found.

In marked contrast to the above is the comparative rapidity with which the excess lactic acid disappears from the blood. After exercise at the rate of 20 and 60 a minute respectively the blood lactic acid has returned to normal after 1 hour of recovery.

TABLE III.

Total Lactic Acid in Rat before and after Exercise.

Controls.		Immediately after 180 per min. for 10 min.		2 hrs. after 180 per min. for 10 min.	
Weight.	Lactic acid.	Weight.	Lactic acid.	Weight.	Lactic acid.
gm.	*mg. per 100 gm. body weight*	*gm.*	*mg. per 100 gm. body weight*	*gm.*	*mg. per 100 gm. body weight*
125	99	109	171	157	120
122	117	115	193	229	115
153	110	154	206	231	121
174	104	140	183	250	146
171	80	164	236	$\overline{219}$	$\overline{126} \pm 14$
136	118	157	224		
119	126	$\overline{140}$	$\overline{202} \pm 24$		
172	110				
177	125				
194	93				
121	111				
$\overline{151}$	$\overline{108} \pm 17$				

The possibility still remains that although the blood lactic acid is normal yet a great deal of the lactic acid that escaped into the blood has rediffused into the muscles and other tissues, and while not yet transformed again into glycogen is held there either as lactic acid or some intermediary substance.

To examine this possibility a further series of rats were exercised at 180 a minute for 10 minutes and after 2 hours recovery were killed and the whole body, including the skin and viscera, was analyzed for lactic acid.   It is necessary to examine the whole carcass in these cases since lactic acid is a freely diffusible substance and probably distributes itself more or less evenly through-

out the various tissues. The results are given in Table III. The controls averaged 108 ± 14 mg. per 100 gm. of body weight. Immediately after exercise the lactic acid content was 202 mg. ± 24 mg. per 100 gm. of body weight. The increase in lactic acid in the whole animal was therefore 94 mg. per 100 gm. of body weight. If we assume that the lactic acid could come into diffusion equilibrium with half the body weight then the apparent blood lactic acid would be about 200 mg. per cent—a value which would appear to be near the maximum for any exercise, voluntary or artificial (cf. Hill, Long, and Lupton (16)). If the muscles retained all the lactic acid formed within themselves, then at the end of exercise their content would be about 0.24 per cent, a value which is about the maximum observed by Meyerhof in the isolated frog muscles stimulated to fatigue, and is also of the same order as that calculated by Hill, Long, and Lupton (16) for man after very severe exercise. Katz and Long (17) observed a maximum of 0.25 per cent for cat gastrocnemius muscle when stimulated to fatigue with the circulation cut off.

2 hours after exercise the average lactic acid content of the whole animal was 126 mg. ± 14 mg. per 100 gm. of body weight, a difference from the resting values that is within the error of the method used. The study of the total lactic acid content of the body leads us to the same conclusion as before, that the removal of excess lactic acid from the body is completed before the muscle glycogen has reached a constant level again. The inference of others that a short time after exercise the blood lactic acid content is an approximate measure of the total lactic acid content of the body would also seem to be correct.

4. Liver Glycogen during and after Exercise.

Since the demonstration by Cori and Cori (6) that d-lactic acid forms liver glycogen with great ease it was of interest to determine the changes, if any, in this substance during and after exercise. At this time the body is flooded with a large amount of lactic acid and the blood perfusing the liver has therefore a greatly increased content of this substance. Himwich, Koskoff, and Nahun (10) observed that in the majority of cases after exercise the blood in passing through the liver lost a greater amount of its lactic acid than it did on passing through any other organ. At

the same time the liver added glucose to the blood. Whether this glucose came from the transformation of lactic acid to glycogen or from preexisting glycogen stores could not be determined.

Our results in Table II show that at two speeds (20 and 60 per minute) the liver glycogen which is initially low had decreased immediately after exercise. This decrease was followed by a slight increase in the next 2 hours. At the third speed observed (180 a minute) the liver glycogen had risen after exercise and then decreased below the preexercise level. The changes however were so small that they are not very significant especially as in our experiments the resting liver glycogen had a wide variation, although it was so low that any marked increases should have been easily detectable. In these experiments if the liver did convert the blood lactic acid into glycogen, this glycogen was not stored but apparently broke down again into glucose and reentered the blood stream.

The blood sugar changes were much more definite. Immediately after exercise at both the speeds examined (20 and 60) the blood sugar had fallen about 30 per cent. Within an hour after the exercise it was normal again.[2]

Levine, Gordon, and Derick (18) showed in man that after marathon races, when the runners were probably in the postabsorptive state, the blood sugar was often much lowered. Hetzel and Long (19) found in fasting diabetics that a bout of exercise lowered the blood sugar.

DISCUSSION.

The chief findings reported in this paper are: (1) the comparatively slow rate at which glycogen is laid down again in the muscles after this artificial exercise; (2) the rapid removal of the excess lactic acid from the blood; (3) the changes in liver glycogen during and after exercise are so small that they are insignificant; (4) the fall and rapid return of the blood sugar to normal.

From previous work we had expected that the resynthesis of glycogen would run parallel with the removal of lactic acid. In

[2] Since the preexisting stores of carbohydrate in the liver could not supply this amount of glucose the source of it must have been protein or lactic acid. There are ample amounts of lactic acid in the body fluids at the end of exercise to supply this quantity of glucose.

these studies on the recovery process in the isolated frog muscles Meyerhof allowed recovery periods from 20 to 44 hours and from such studies he calculated the oxidation quotient. Recovery may have been complete in a shorter time but he gives no experiments to support this. In the intact mammal the higher body temperature and the very efficient circulation would lead one to expect that a more rapid rate of glycogen resynthesis than we have found would exist. On the other hand the rate of removal of lactic acid after exercise is very rapid in all mammals that have been studied. In man as well as in the rat it is complete in 1 to 2 hours.

It is too early to attempt to fit these results into any general scheme of the recovery process in the mammal and for the present we wish to confine ourselves to a statement of them.

From this and previous work any such scheme must take into consideration the fact that lactic acid escaping from the muscles during exercise may be converted into liver glycogen, and from this point its disposal would be that of glucose. Under the conditions existing during recovery the greater part of this glucose may be oxidized and only a small portion deposited as muscle glycogen or some intermediary substance. The importance of the quantity of glycogen already existing in the liver at the time exercise is undertaken must also be considered in any interpretation of the substances responsible for muscle glycogen resynthesis during recovery in the intact mammal.

SUMMARY.

1. A method has been devised for exercising 24 hour fasted rats so that the glycogen content of the whole body is reduced by fairly constant amounts.

2. By this method it has been possible to show: (a) that after this type of exercise the restoration of the body glycogen is a slow process requiring about 12 hours for its completion; (b) the removal of lactic acid from the blood and body is much more rapid, requiring about 1 to 2 hours for its completion; (c) the glycogen content of the liver is not greatly altered by exercise or during recovery; (d) the blood sugar falls during exercise, but is rapidly restored to normal.

We have to thank Dr. C. F. Cori for his advice on certain points of technique. We are also indebted to Dr. J. B. Scriver for supplying us with rats from her colony.

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METABOLISM OF AMINES.

II. DIMETHYLAMINE.

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Questions which have arisen during a recent study of the me‑ tabolism of trimethylamine by rabbits (1) have made desirable a similar study of the metabolism of dimethylamine. Trimethylamine, when it is metabolized, loses its methyl groups, and dimethylamine in small amount, together with urea, equivalent at least to the amine which disappears, are excreted. The significance of the occurrence of the small amounts of dimethylamine cannot be interpreted without information as to the changes which dimethylamine itself may undergo in the body of the rabbit. If the methyl groups of trimethylamine are removed one at a time, and at equal rates, a longer time should be required for its metabolism than for dimethylamine, so that after ingestion by a given animal of similar amounts of the bases, relatively more unchanged trimethylamine than dimethylamine might be recovered in the urine. If, however, the methyl groups are removed at quite different rates, the above expectancy might not be fulfilled, and dimethylamine might be excreted in larger amounts than is trimethylamine.

The biochemical process by which methyl groups are removed from an atom of nitrogen is probably either through oxidation of methyl to carboxyl groups, with subsequent decarboxylation, or else through hydrolysis directly to methyl alcohol and the amine having fewer methyl groups. Of the two processes, the second only is likely to be reversible. Therefore, were trimethylamine excreted in the urine after ingestion of dimethylamine, good evi-

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* Technical assistance was given by Margaret Rowntree.

dence as to the chemical nature of the demethylation process would be available.

An understanding of the biological transformations of many substances cannot be made complete without adequate data concerning the transformations of dimethylamine in the body. Part of the nitrogen of any quaternary ammonium base, betaine, tertiary amine, and of most secondary amines, probably is converted at some stage in its metabolism to dimethylamine. Sarcosine (from creatine) and perhaps adrenalin, are common substances which would be expected to liberate part of their nitrogen directly in this form.

Previous biological studies with dimethylamine have dealt with either the occurrence of dimethylamine as the result of putrefactive processes or else with the pharmacological properties of the base. Quantitative data upon the excretion of the amine after its ingestion have not been found.

The procedure followed in the present work was essentially the same as that already described (1). Rabbits were maintained upon a diet of carrots exclusively and the daily excretion of nitrogen was brought to a value which was less than 1 gm. per day, and which varied only slightly. By use of a stomach tube, the amine was given as a solution of the hydrochloride, followed immediately by a corresponding amount of sodium carbonate in solution. Benzoic acid was added to the bottles for collecting the urine in order to prevent ammoniacal decomposition and loss of volatile base. The urine was diluted to 500 cc. and 50 cc. portions were removed for the determination of total nitrogen. The remainder was made alkaline with magnesium oxide, and about 300 cc. were distilled off *in vacuo* into excess of standard acid. The volatile base thus obtained was analyzed for ammonia, trimethylamine, and dimethylamine (2).

The analytical data are presented in Tables I to IV, in which results with six rabbits are shown. Rabbits 1, 2, and 3 had been used in studies with trimethylamine (1), and the experiments reported here (Table I) are comparable with those studies. Upon comparison of this table with those for trimethylamine it becomes evident that the percentage recovery of unchanged amine was greater for dimethylamine than for trimethylamine. It seems that either dimethylamine is metabolized more slowly

than is trimethylamine, or else is excreted more rapidly. The excretion of some dimethylamine after ingestion of trimethylamine,

TABLE I.

Recovery of Amines from Urine of Rabbits after Ingestion of Dimethylamine.

Date	Weight	N intake	N output	Base	Amines	NH_3	$(CH_3)_3N$	$(CH_3)_3N$	$(CH_3)_2NH$	$(CH_3)_3NH$	$(CH_3)_2NH$ recovery
1929	gm.	gm.	gm.	cc. N acid	cc. N acid	gm.	cc. N acid	gm.	cc. N acid	gm.	per cent
Rabbit 1, female.											
Mar. 18		0.528	1.176	0.744	0.086	0.011					
" 19	3120	0.528	0.084	0.119	0.046	0.001					
" 20		0.614	0.880	3.357	1.266	0.036	0.032	0.002	0.528	0.025	9.0*
" 21		0.614	0.863	1.361	1.144	0.003	0.030	0.002	1.260	0.058	21.0*
" 22		0.462	0.921	6.824	4.062	0.046	0.027	0.002	3.800	0.171	30.9†
" 23	2935	0.528	0.798	0.334	0.289	0.007	0.009	0.000	0.075	0.003	
" 24		0.528	0.773	0.128							
Rabbit 2, male.											
Mar. 18		0.510	0.590	1.187	0.047	0 019					
" 19	2705	0.501	0.581	1.445	0.049	0.024					
" 20		0.367	0.662	1.961	1.413	0.009	0.014	0.001	1.568	0.071	25.7*
" 21		0.297	0.876	0.795	0.386	0.007	0.016	0.001	0.400	0.018	6.5*
" 22		0.277	1.011	3.192	2.443	0.013	0.020	0.001	2.565	0.116	21.0†
" 23	2600	0.237	0.721	0.736	0.200	0.009	0.010	0.000	0.180	0.008	
" 24		0.510	0.429	0.059							
Rabbit 3, male.											
Mar. 18		0.528	0.665	0.353	0.081	0.059					
" 19	2640	0.528	0.567	0.351	0.077	0.041					
" 20		0.614	0.587	0.214	0.092	0.019	0.012	0.001	0.065	0.003	1.0*
" 21		0.614	0.681	0.878	0.681	0.033	0.010	0.001	0.634	0.028	10.1*
" 22		0.699	0.628	2.798	2.174	0.012	0.005	0.000	1.433	0.064	11.5†
" 23	2445	0.528	0.284	0.182	0.173	0.000	0.034	0.002	0.138	0.006	
" 24		0.528	0.717	0.150							

* 0.500 gm. of $(CH_3)_2NH \cdot HCl$ and 0.200 gm. of Na_2CO_3 were given.

† 1.000 gm. of $(CH_3)_2NH \cdot HCl$ and 0.400 gm. of Na_2CO_3 were given.

therefore, is to be expected if it is formed from trimethylamine. A more rigid comparison of the metabolism of the two amines is

contemplated.　Table I illustrates also that there is no appreciable delay in the excretion of the amine, 90 per cent or more of it being eliminated within 24 hours.

More complete data upon the excretion of dimethylamine were obtained with Rabbits 5, 6, and 7 (Tables II and III).　These experiments differed from those in Table I in that twice the amount of sodium carbonate (2 chemical equivalents instead of 1) was administered with the amine hydrochloride.　This difference was about 0.12 gm. of sodium carbonate per day per kilo of rabbit in excess over that required to liberate the amine from the hydrochloride.　The total dosage was considered to be 0.290 gm. of free dimethylamine, and the chemically equivalent amounts of NaCl (0.37 gm.) and of $NaHCO_3$ (0.537 gm.) per day for each rabbit.　This large amount of amine given together with the extra equivalent of base seemed to bring about an excretion of amine greater in amount than had been noted previously.　This was desirable because with more complete excretion of amine, trimethylamine and monomethylamine, if they were formed from dimethylamine, also should be excreted in larger amounts.　One experiment is presented in detail in Table II, and the average values for the experiment are given in Table III in such a way that they may be compared readily with averages from a similar experiment, the details of which are not presented.　The rabbits, with one exception (Rabbit 6) seemed always to be in good physical condition, but exhibited an abnormal nervous response in resisting the daily massage of the bladder.　Rabbit 6 ate only part of its ration each day, and lost much in weight.

The data indicate that in general, about 50 per cent of the dimethylamine is excreted unchanged.　The excretion of this amine seems to be independent of the nitrogen intake, as well as of the excretion of ammonia.　For the reason, however, that the excess of non-volatile base seemed to bring about a marked increase .in the excretion of the amine, further studies upon the effect of ingestion of alkali upon the excretion of the amines will be made.

In order to prove that the secondary amine which was recovered was dimethylamine, it was converted into the p-bromobenzenesulfonamide by heating in NaOH solution with p-bromobenzenesulfochloride.　The product which separated was crystallized from dilute alcohol, whereupon it melted at 92–92.5°.　A similar pro-

TABLE II.

Recovery of Amines from Urine of Rabbits after Ingestion of Dimethylamine.

Date.	Weight.	N intake.	N output.	Base.	Amines.	NH₃	(CH₃)₃N	(CH₃)₃N	(CH₃)₂NH	(CH₃)₂NH	(CH₃)₂NH recovery.
1930	gm.	gm.	gm.	cc. N acid	cc. N acid	gm.	cc. N acid	gm.	cc. N acid	gm.	per cent

Rabbit 5, female.

Date.	Weight.	N intake.	N output.	Base.	Amines.	NH₃	(CH₃)₃N	(CH₃)₃N	(CH₃)₂NH	(CH₃)₂NH	(CH₃)₂NH recovery.
July 15		0.528	0.567	3.718	0.313						
" 16	2560	0.618	0.634	5.131	3.777	0.023	0.025	0.002	3.625	0.163	56.2*
" 17		0.618	0.590	5.225	3.887	0.023	0.005	0.000	3.565	0.160	55.3*
" 18		0.618	0.483	8.855	3.820	0.086	0.013	0.001	3.205	0.144	49.7*
" 19		0.618	0.518	4.191	2.860	0.023	0.025	0.002	2.602	0.117	40.4*
" 20		0.618	0.490	5.478	4.237	0.021	0.005	0.000	3.923	0.176	60.9*
" 21		0.618	0.581	5.511	3.500	0.034	0.015	0.001	3.045	0.137	47.6*
" 22		0.618	0.504	7.599	3.032	0.078	0.001	0.000	2.839	0.128	44.0*
" 23	2568	0.528	0.436	3.526	0.420	0.048	0.008	0.000	0.360	0.016	
" 24		0.528	0.490	11.473	0.052	0.194	0.005	0.000	0.037	0.002	

Rabbit 6, female.

Date.	Weight.	N intake.	N output.	Base.	Amines.	NH₃	(CH₃)₃N	(CH₃)₃N	(CH₃)₂NH	(CH₃)₂NH	(CH₃)₂NH recovery.
July 15		0.528	0.410	2.717	0.338						
" 16	2382	0.618	0.488	2.805	1.992	0.014	0.023	0.002	1.870	0.084	29.0*
" 17		0.194	0.581	4.279	3.200	0.018	0.013	0.001	3.062	0.138	47.5*
" 18		0.273	0.747	21.120	1.987	0.325	0.008	0.001	1.910	0.086	29.6*
" 19		0.195	0.770	3.773	3.330	0.008	0.018	0.001	3.142	0.141	48.7*
" 20		0.136	0.798	6.875	3.800	0.052	0.015	0.001	3.267	0.147	50.6*
" 21		0.125	0.875	6.110	3.000	0.053	0.001	0.000	2.758	0.124	42.8*
" 22		0.148	0.809	3.564	3.062	0.008	0.020	0.001	2.785	0.125	43.1*
" 23	2147	0.229	0.742	1.914	0.372	0.025	0.015	0.001	0.312	0.014	
" 24		0.528	0.701	20.262	0.025	0.342	0.005	0.000	0.017	0.008	

Rabbit 7, female.

Date.	Weight.	N intake.	N output.	Base.	Amines.	NH₃	(CH₃)₃N	(CH₃)₃N	(CH₃)₂NH	(CH₃)₂NH	(CH₃)₂NH recovery.
July 15		0.352	0.480	0.352	0.303						
" 16	2243	0.442	0.597	3.179	2.647	0.009	0.021	0.001	2.433	0.109	36.7*
" 17		0.442	0.567	5.233	4.344	0.015	0.018	0.001	3.842	0.173	59.6*
" 18		0.442	0.484	5.346	4.287	0.018	0.010	0.001	3.710	0.167	57.5*
" 19		0.442	0.560	3.229	2.812	0.007	0.015	0.001	2.680	0.120	41.6*
" 20		0.442	0.532	6.275	4.800	0.025	0.030	0.002	4.165	0.187	64.6*
" 21		0.442	0.504	4.983	4.262	0.011	0.024	0.002	3.715	0.167	57.6*
" 22		0.322	0.673	5.137	3.627	0.026	0.006	0.000	3.228	0.145	50.1*
" 23	2176	0.352	0.568	0.990	0.415	0.010	0.005	0.000	0.349	0.016	
" 24		0.352	0.532	4.069	0.072	0.068	0.003	0.000	0.053	0.002	

* 0.525 gm. of (CH₃)₂ NH·HCl and 0.600 gm. of Na₂CO₃ were given.

duct prepared from dimethylamine hydrochloride melted at 91°, and mixed with the first mentioned product melted at 91–92.5°. The substances were therefore identical, and were *p*-bromobenzenesulfodimethylamide.

The amounts of trimethylamine which are reported in the tables are considered to be within the limits of error of the method used for the analyses. Similarly, calculations of the amounts of

TABLE III.

Recovery of Amines from Urine of Rabbits after Ingestion of Dimethylamine.
Averages for 7 day periods.

Date.	N intake.	N output.	Base.	Amines.	NH_3	$(CH_3)_3N$	$(CH_3)_3N$	$(CH_3)_2NH$	$(CH_3)_2NH$	$(CH_3)_2NH$ recovery.
Rabbit 5, female.										
1930	*gm.*	*gm.*	*cc. N acid*	*cc. N acid*	*gm.*	*cc. N acid*	*gm.*	*cc. N acid*	*gm.*	*per cent*
June 25–July 2	0.561	0.681	5.291	3.378	0.032	0.015	0.001	3.029	0.136	46.7*
July 16–23	0.618	0.543	5.999	3.586	0.035	0.010	0.001	3.272	0.147	50.6*
Rabbit 6, female.										
June 25–July 2	0 566	0.625	7.300	3.698	0.051	0.016	0.001	4.238	0.169	58.0*
July 16–23	0.241	0.724	6.932	2.910	0.065	0.013	0.001	2.865	0.120	41.6*
Rabbit 7, female.										
June 25–July 2	0.426	0.665	5.370	3.882	0.021	0.019	0.001	3.208	0.151	51.7*
July 16–23	0.425	0.559	4.770	3.825	0.016	0.017	0.001	3.396	0.153	52.5*

* 0.524 gm. of $(CH_3)_2NH \cdot HCl$ and 0.600 gm. of Na_2CO_3 were given each day.

monomethylamine which were excreted give values which are not greater than experimental error. These experiments indicate, therefore, that methylation of dimethylamine to trimethylamine does not take place to an appreciable extent. They do not prove, however, that the process of demethylation is completely irreversible.

The excretion of ammonia again was found to vary greatly, as may be seen from Table II, and there were occasional values which

were abnormally high. It was expected that the amount of ammonia excreted on the days of the experiment would be less than

TABLE IV.

Excretion of Urea and of Ammonia and Amines after Ingestion of Dimethylamine.

Date.	N intake.	N output.	Volatile base plus urea N.	Urea N.	Volatile base N.
			Rabbit 5, female.		
1930	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
July 26	0.528	0.616	0.469	0.462	0.007
" 27	0.528	0.616	0.455	0.447	0.008
" 28	0.614	0.686	0.490	0.420	0.070*
" 29	0.614	0.672	0.489	0.469	0.020*
" 30	0.614	0.805	0.688	0.598	0.090*
" 31	0.528	0.658	0.463	0.455	0.008
Aug. 1	0.528	0.599	0.458	0.453	0.005
			Rabbit 6, female.		
July 26	0.097	0.267	0.217	0.203	0.014
" 27	0.249	0.476	0.336	0.336	0.000
" 28	0.145	1.008	0.760	0.750	0.010*
" 29	0.131	0.987	0.756	0.679	0.077*
" 30	0.167	1.104	0.826	0.706	0.120*
" 31	0.306	0.700	0.488	0.468	0.020
Aug. 1	0.176	0.746	0.530	0.523	0.007
			Rabbit 7, female.		
July 26	0.352	0.490	0.371	0.364	0.007
" 27	0.352	0.585	0.434	0.424	0.010
" 28	0.438	0.742	0.482	0.478	0.004*
" 29	0.438	0.728	0.560	0.490	0.070*
" 30	0.438	0.707	0.609	0.516	0.093*
" 31	0.352	0.588	0.436	0.428	0.008
Aug. 1	0.352	0.502	0.392	0.385	0.007

* 0.498 gm. of $(CH_3)_2NH \cdot HCl$ and 0.600 gm. of Na_2CO_3 were given.

on the other days, since the ingestion of $NaHCO_3$ usually diminishes the amount of ammonia in the urine. At no time during the experiments were the precautions to prevent ammoniacal de-

composition of the urine omitted, nor were the urines kept beyond the few hours required for the distillations.   The degree of evacuation of the distillation flask was always checked by use of a manometer, and the temperature used was never sufficiently high to decompose urea by ordinary hydrolysis.

The effect of the ingestion of dimethylamine upon the excretion of urea is shown in Table IV.   In two out of three cases there was an unquestionable increase in the urea output when the amine was administered.

SUMMARY.

Dimethylamine, when fed to rabbits in amounts from 92 to 220 mg. of free base per day per kilo of rabbit is metabolized to the extent of 70 to 99 per cent, the remainder being excreted as the unchanged amine.   When fed at the rate of about 130 mg. per day per kilo with an equivalent amount of non-volatile base, it is metabolized only to the extent of 43 to 71 per cent of the amine ingested.

Trimethylamine and monomethylamine are not excreted in appreciable amounts after the ingestion of dimethylamine.   The process of demethylation of the nitrogen of trimethylamine seems therefore not to be readily reversible.

The excretion of ammonia seems to be independent of the amount of dimethylamine metabolized.

Apart from that which may give rise to ammonia, most if not all, of the nitrogen of dimethylamine which is metabolized seems to be excreted as urea.

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OBSERVATIONS ON THE SERUM CALCIUM, PROTEINS, AND INORGANIC PHOSPHORUS IN EXPERIMENTAL VITAMIN B DEFICIENCY AND INANITION.*

By VICTOR SCHELLING.

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In recent experimental vitamin B deficiency studies the usual practice now is to provide the test animals with a complete diet containing all essentials in proper proportion, omitting only the factor under investigation.   Such a program is especially valuable for physiological experiments.   Agreement in the observations on different species of animals can only be expected when the nutritional conditions are equivalent.   Many controversies are found in the earlier literature on experimental vitamin B deficiency due probably to the disregard of an incomplete diet and the abstinence of food which animals on a vitamin B-free diet frequently exhibit. Thus the convulsions and, in the most typical cases, the head retraction of pigeons fed on a polished rice diet have been considered the most characteristic symptoms of the vitamin B deficiency. Since this state is also obtained by prolonged fasting, a more exhaustive investigation seemed essential to determine the vitamin B deficiency effect.   McCarrison (12), Drummond (4), Kon and Drummond (8), and many others have studied extensively the different factors collectively responsible for what is called a vitamin B-deficient state.   Today we fortunately have very good combinations of diets for the three species of animals most used in these experiments, namely the pigeon, the rat, and the dog.

In regard to the blood calcium level in experimental B avitaminosis, two different findings are reported.   Ungar (20), working on pigeons, assumed a relation between the convulsions and the blood calcium level.   Upon determination the serum calcium was found

* Throughout this paper vitamin B refers to the whole complex B_1, B_2, and B_3.

not to be altered in beriberi pigeons. Similar results were obtained with pigeons by Kon and Drummond (8), who observed a rather slight increase. On the other hand Smith (19) states that "Blood calcium determinations upon dogs in spastic paralysis from vitamin B deficient diets ranged from 7.25 to 7.45 mgm. per 100 cc. This is near the tetany level."

Because of this divergence of data, the present research has been undertaken. In addition to the serum calcium, the proteins and the inorganic phosphorus were determined. It is concluded from recent studies (Marrack and Thacker (11), Loeb and Nichols (10), Greenberg and Gunther (5), and others) that the blood calcium is partly in a diffusible ionized form and partly in a non-diffusible colloidal form, probably bound to plasma proteins. From their physicochemical observations of the solubility of $CaCO_3$ in blood serum, Hastings, Murray, and Sendroy (6) derived a linear equation for the relationship of the calcium to the proteins. Furthermore, Sendroy and Hastings (18) observed a decrease in the solubility of $CaCO_3$ in serum in the presence of phosphates. Recently, Peters and Eiserson (17) have brought these relations into a formula which they derived from clinical cases.

<div align="center">EXPERIMENTAL.</div>

Animals.—Dogs were used for test animals. Fully grown dogs weighing from 8 to 11 kilos were kept on the artificial diet suggested by Karr (7) and improved by Cowgill (3) composed of casein 63 gm., sucrose 5.84 gm., lard 2.83 gm., butter fat 1.17 gm., agar 0.40 gm., and salt mixture[1] 0.20 gm. per kilo unit of body weight. In addition to the dietary factors mentioned in these papers, 3 to 5 cc. of cod liver oil (Parke, Davis and Company) were supplemented daily as a source of vitamins A and D. For the vitamin B-free diet the casein was extracted twice with acidulated water and washed on the suction funnel with 50 per cent alcohol, then dried by spraying out on filter paper, and finally heated in the autoclave for 3 hours at a steam pressure of 15 pounds. From our experience it seemed advisable to keep the dogs for a period of 2 to 3 weeks on this diet supplemented with yeast. Because it is rather difficult to provide the dogs for a longer time with a meat-free diet, this

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[1] The salt mixture was that of Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **32**, 317 (1917).

preceding observation time allows one to choose those animals which feed voluntarily. It should be stressed that the animals consume the diet regularly. Vitamin B acts as a strong stimulant for the appetite, therefore on a vitamin B-free diet it was frequently found that the animals refused food after a considerable preliminary period of satisfactory food intake. From the moment of refusing food an increased loss of weight was noted, followed by paralysis after 10 to 15 days of fasting. Such an inanition period implies of course a multiple vitamin deficiency. However, the findings should not be confused with those of the uncomplicated vitamin B-deficient state. This work proves again that it is possible to feed dogs successfully with a meat-free diet for a period of 98 days.

Methods.—The blood calcium was estimated in the first experiments by the method of Clark and Collip (2) and later the new method of Van Slyke and Sendroy (21) was adopted. Because the gasometric determination of CO_2 from the calcium oxalate is somewhat time-consuming, the oxalate was titrated with 0.01 N potassium permanganate, with a color standard and the precautions outlined for the permanganate micro titrations by Mika (13). The inorganic phosphorus was estimated by the method of Benedict and Theis (1). The proteins were determined by a micro-Kjeldahl method with the apparatus of Parnas and Wagner (16) the corresponding figures for the non-protein nitrogen being taken into account.

The blood was collected from the fasting animal without an anticoagulant in an ice-cooled test-tube and allowed to remain in ice for 1 hour. The clear serum, centrifuged at a speed of 2500 revolutions, was used for the determinations. Curves I, Chart I, illustrate the findings on a dog fed for a period of 98 days on a'vitamin-free diet. There was a slight decrease in the calcium from 13.7 mg. to 12 mg. and the phosphorus from 4.6 mg. to 3.9 mg. during the first 3 weeks, probably due to the radical change in the nutritional conditions. After this the calcium remained at the same level for a remarkably long time. Phosphorus and proteins did not show any considerable variations during the experiments. Curves II and III represent the state of vitamin B deficiency followed by that of fasting. Dog 52 (Curves II) was kept for 3 weeks on the vitamin B-free diet, after which time he discontinued

to eat.   A rapidly increasing loss of body weight followed, with a marked degree of apathy.   On the 6th day of fasting he developed paralysis of the legs and they were cold to touch.   The total loss of body weight amounted to 26 per cent of the original weight.   On the 10th day of the vitamin-free period a drop in the calcium oc-
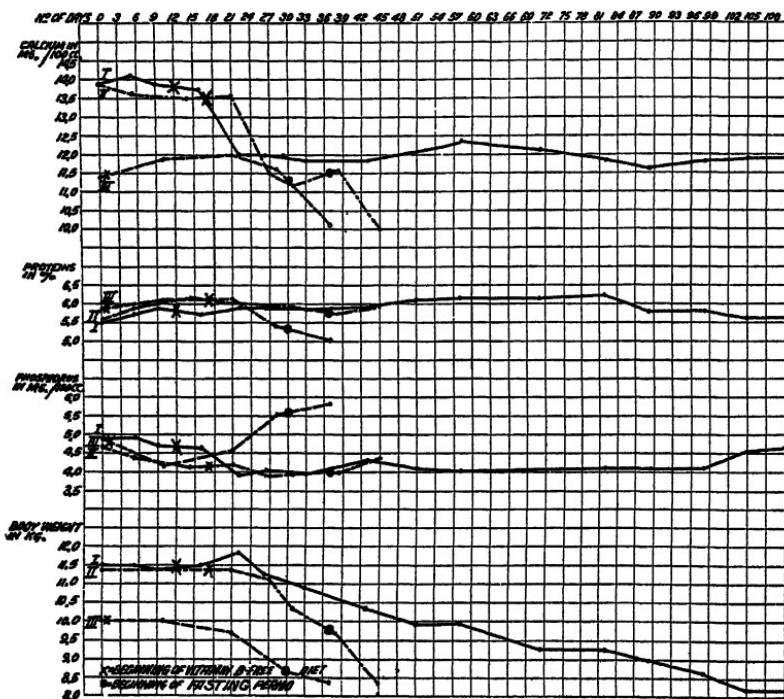
CHART I. Serum calcium, proteins, and inorganic phosphorus in vitamin B deficiency and inanition.   Dog 51 was kept for 98 days on a vitamin B-free diet.   Dog 52 was kept for 20 days on a vitamin B-free diet.   After that time the food was refused.   Animal died on the 8th day of fasting.   Dog 54 was kept 29 days on the vitamin B-free diet, followed by a fasting period of 9 days.

curred and again in the final fasting state.   The proteins did not show any remarkable change while the phosphates slightly increased.   Unable to take any food by mouth the dog was given 500 cc. of milk mixed with 50 gm. of yeast powder (Harris) by means of the stomach tube, after which it recovered quickly.

Curves III give the experimental results on a dog which discontinued to eat after the 4th week on the vitamin B-free diet. The total loss of body weight reached 27 per cent. In this case a decrease of the calcium was also observed, but the increase of the inorganic phosphorus was more marked. A slight decrease in the proteins occurred.

DISCUSSION.

From the above observations it is concluded that the serum calcium does not show any remarkable decrease during the vitamin B-free period. The convulsions in tetany are accompanied by an increased drop in the serum calcium. Therefore it may be assumed that the convulsions observed in the final stage of vitamin B deficiency must be of different origin.

The discrepancy in the data of Ungar (20) and Smith (19) regarding the blood calcium level is undoubtedly due to divergent nutritional conditions. Ungar kept his pigeons on a polished rice diet. This mode of feeding, besides being inadequate in several respects, sooner or later leads to fasting. Smith used the diet suggested by Karr (7) for her experiments on dogs. In these experiments the same diet was used but we are unable to corroborate her findings. Neither in the uncomplicated vitamin B-deficient state nor in the complex state of fasting have we observed figures so low as 7 mg. The decrease in calcium observed in these experiments is in accord with the findings of Morgulis (14) on completely fasted dogs. It begins when the loss of body weight exceeds 20 per cent.

The total serum nitrogen as well as the non-protein nitrogen was increased in all cases of human beriberi reported by Kozawa, Kusunoki, and Hosoda (9). Contrary to these findings are the experimental observations on pigeons by Palmer and Hoffman (15) who found a decrease in both the total nitrogen and the non-protein nitrogen. As Chart I shows, only a slight variation in the proteins occurred in our experiments. Practically the proteins remained at the same level.

So far as the inorganic phosphorus is concerned, no reference can be found in the literature related to the experimental vitamin B deficiency.

SUMMARY.

1. Data are presented for the calcium, proteins, and inorganic phosphorus in the blood of dogs deprived of vitamin B (whole complex) and of fasted dogs.

2. With one exception where the calcium dropped from 13.6 mg. to 11.5 mg. no remarkable decrease in the calcium during the vitamin B-free period was observed.

3. There is no noteworthy change in the proteins and the inorganic phosphorus.

4. In fasting dogs a decrease in the calcium was observed in the advanced state of paralysis when the loss of body weight exceeded 20 per cent; the inorganic phosphorus was found to be increased; in one case the proteins were slightly decreased.

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A COMPARISON OF THE INFLUENCE OF IODIZED MILK AND OF POTASSIUM IODIDE ADMINISTERED DIRECTLY, ON THE SIZE AND IODINE CONTENT OF THE THYROID GLAND OF RATS.

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PLATE 1.

It is now an established fact that the iodine content of milk can be materially increased by feeding cows some source of inorganic iodine, such as potassium iodide, or some source of organic iodine, such as kelp. Considerable milk containing more than a normal amount of iodine is probably being produced through the feeding of iodized mineral mixtures and commercial preparations made up of iodine-containing substances. It is also well known that the iodine content of milk may vary from a trace to several parts per million, depending upon regional influences.

In view of the therapeutic value of iodine in the treatment of simple goiter, the question arose as to the effectiveness of iodized milk in this respect. Also, in view of the fact that supplemental iodine in one form or another is being fed to cows for benefit to the cows themselves, it seemed well to determine whether the iodized milk so produced would be harmful or beneficial. The study here reported was undertaken to obtain information bearing on this question.

Part I.

Albino rats from our own colony were used throughout and were fed nothing but milk to which were added sufficient iron and copper to prevent nutritional anemia from developing. This system of feeding was adopted in order to limit the iodine supply to one source and to prevent variations that occur when an ordinary basal ration is used. The milk was fed in glass beakers and the

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amount consumed was accurately measured.   When additions of potassium iodide were made, a small enough quantity of milk was fed in the evening to insure quantitative consumption of the added iodine; the following morning the balance of the 50 cc. daily allowance of milk was fed.

At the close of each trial the rats were anesthetized with ether and killed by bleeding from the carotid artery.   The amount of inorganic phosphorus in this blood was determined by the method of Fiske and Subbarow (1).   The two lobes of the thyroid gland were removed, weighed immediately, then dried and weighed again. The dried glands of an entire group, or of the males and females separately, were combined to make a large enough sample for determining iodine by the same method used in the milk analyses (2), except that the extractions with alcohol were omitted.   The femurs were removed, extracted with alcohol and ether, and their ash content determined.

Two Jersey cows fed the usual herd ration furnished the milk designated as normal; two Jersey cows fed the same ration, to the grain.mixture of which was added daily 0.1 gm. of potassium iodide, furnished the milk designated as iodized.   Samples were obtained daily by collecting aliquots of the morning and evening milkings and combining the aliquots from each cow in equal volumes.   Analyses by the method of Leach and Henderson (2) revealed no iodine in the normal milk; while the iodized milk was found to contain approximately 5 parts of iodine in 10 million.

Trial 1.—This consisted of two series, but inasmuch as the conditions were identical in each series, the data obtained have been combined and treated as a single trial.   Six groups of rats were used.   Group I received normal milk; Group II, normal milk plus 0.000025 gm. of iodine as potassium iodide; Group III, normal milk plus 0.00005 gm. of iodine as potassium iodide; Group IV, normal milk plus 0.0000125 gm. of iodine as potassium iodide; Group V, iodized milk; and Group VI, iodized milk diluted with an equal volume of normal milk.   The duration of this trial was 12 weeks.

Trial 2.—In this trial, normal milk, iodized milk, and a combination of half iodized and half normal milk were compared over a period of 14 weeks.

Trial 3.—The same general procedure used in Trials 1 and 2

was followed in Trial 3. However, in order to overcome any effect of individuality that may have influenced the results obtained in Trials 1 and 2, the cows were reversed; *i.e.*, those receiv-

TABLE I.

Effect of Potassium Iodide and of Iodized Milk on Size and Iodine Content of Thyroid Gland of Rats.

Group.	No. of rats.	Body weight.	Weight of fresh thyroid.	Weight of dry thyroid.	Gland weight (fresh) per 100 gm. body weight.	Gland weight (dry) per 100 gm. body weight.	Iodine in dry gland.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
Series I (12 wks.).							
Normal milk.................	7	158	0.0088	0.0022	0.0056	0.0014	0.063*
" " + 0.000025 gm. I₂.	8	163	0.0079	0.0023	0.0049	0.0014	0.431†
" " + 0.00005 " "	5	159	0.0085	0.0017	0.0054	0.0011	0.672
" " + 0.0000125 " "	4	162	0.0122	0.0022	0.0075	0.0014	0.279
Iodized " , 50 cc. = approximately 0.000025 gm. I₂.......	7	144	0.0078	0.0021	0.0054	0.0015	0.511*
½ iodized milk, ½ normal milk...	5	162	0.0087	0.0023	0.0054	0.0015	0.597
Series II (14 wks.).							
Normal milk.................	4	158	0.0081	0.0014	0.0051	0.0009	0.139
Iodized "	3	135	0.0059	0.0011	0.0042	0.0008	0.363
½ iodized milk, ½ normal milk....	4	152	0.0051	0.0011	0.0034	0.0007	0.376
Series III (8 wks.).							
Normal milk.................	6	131	0.0110	0.0036	0.0085	0.0028	0.203
" " + 0.000025 gm. I₂..	6	135	0.0089	0.0028	0.0066	0.0020	0.396
Iodized "	6	136	0.0077	0.0023	0.0057	0.0017	0.496
½ iodized milk, ½ normal milk...	6	130	0.0079	0.0024	0.0060	0.0019	0.561

* Based on two rats.
† Based on three rats.

ing iodine during Trials 1 and 2 were "normal" cows during Trial 3, and those fed normally in Trials 1 and 2 were now "iodized" cows. This trial continued for 8 weeks.

The iodine content of the milk was determined at intervals

during the trials. The average iodine content of the milk fed was approximately 0.05 mg. per 100 cc. or 5 parts in 10 million. There was some variation in the iodine content of the milk; the usual range was between 4 and 6 parts per 10 million. The normal milk used in these trials contained no iodine detectable by the method used.

The results obtained in Trials 1, 2, and 3 are presented in Table I. In general, on the basis of fresh weight of the gland, the administration of iodine to rats, either as potassium iodide or as it is combined in milk, resulted in smaller thyroid glands than when nothing but normal milk was fed. Likewise, as the iodine intake increased the iodine content of the thyroid increased. This is in keeping with the theory of the inverse ratio between iodine intake and size of the thyroid gland, and agrees with the work of Evvard (3) on new born lambs.

In each series, dilution of the iodized milk with an equal volume of normal milk gave as good results with respect to size of gland, and increased the percentage of iodine in the gland over that obtained when undiluted iodized milk was fed. This might indicate that the amount of iodine furnished by the iodized milk was in excess of that required for optimum utilization.

While the weight of the fresh gland indicates the size of the gland at a particular time, it does not allow for age or body weight. Since, according to Donaldson (4), the weight of the thyroid gland is almost a linear function of the body weight, the most significant figures bearing on size of the thyroid are those based on fresh weight per 100 gm. of body weight. Considering the data in this way does not alter the inferences based on weight of the fresh gland alone.

Part II.

In preliminary work to perfect the technique of removing thyroid glands from rats, it was noticed that rats fed the high calcium-low phosphorus diet of Steenbock and Black (yellow corn 76, wheat gluten 20, calcium carbonate 3, sodium chloride 1) (5) invariably had larger thyroids than those fed a normal mixed diet or an exclusive milk diet. This suggested a method for studying the curative effect of iodine additions.

Weanling rats were fed the above diet for 4 weeks. Eleven rats

were then killed and disposed of in the aforementioned way; the rest were divided into four groups. Two of the groups were continued on this basal rachitic diet; to each rat of one group 0.000025 gm. of iodine as potassium iodide was given daily. The rats of this and a second group were exposed to ultra-violet light from a Cooper Hewitt quartz mercury lamp three times a week for 10 minutes at 20 inches from the burner. This was done in order to prevent extreme rickets. The rats of the remaining groups were deprived of the basal diet, for which was substituted

TABLE II.

Effect of Addition of Ultra-Violet Radiation, Potassium Iodide, Normal Milk, and Iodized Milk to a Rickets-Producing Diet on the Size and Iodine Content of the Thyroid Gland of Rats.

Group.	No. of rats.	Weight of thyroid (fresh).	Weight of thyroid (dry).	Iodine in dry gland.
		gm.	*gm.*	*per cent*
Stock rats 8 wks. of age.............	4	0.0093	0.0024	0.138
Basal diet* for 4 wks................	11	0.0238	0.0046	0.058
" " for 4 wks., then plus ultra-violet radiation for 4 wks.........	8	0.0228	0.0049	Trace.
Basal diet for 4 wks., then plus ultra-violet radiation and 0.000025 gm. I₂ for 4 wks.....................	10	0.0103	0.0031	0.407
Basal diet for 4 wks., then 50 cc. normal milk for 4 wks..............	9	0.0113	0.0030	0.213
Basal diet for 4 wks., then 50 cc. iodized milk for 4 wks.............	10	0.0099	0.0027	0.510

* Yellow corn 76, wheat gluten 20, calcium carbonate 3, sodium chloride 1.

50 cc. of normal milk in one group and 50 cc. of iodized milk in the other. 4 weeks after the division into groups was made the rats were disposed of as described in Part I.

Two duplicate series were run, but inasmuch as the results for each series were parallel they are combined and presented in Table II. It is apparent that the addition of potassium iodide or iodized milk resulted in a reduction in the size of the thyroid gland and increased the iodine content of this organ.

Figs. 1 to 6 are photographs of the thyroid glands of rats representative of each group used in Part II.

An interesting variation occurred in this trial. A small amount

of iodine was found in the normal milk, averaging 0.015 mg. per 100 cc. The iodized milk was also a little higher in iodine content, averaging 0.067 mg. per 100 cc. The reason for this could not be determined. It is possible that the presence of this amount of iodine in the normal milk prevented greater differences in size and iodine content of the thyroid glands of the rats in the respective normal and iodized milk groups.

<div align="center">DISCUSSION.</div>

The relationship between iodine and the thyroid gland has been thoroughly discussed recently by Orr and Leitch (6) and by Evvard (3). Any repetition here would be superfluous. While it is generally conceded that the administration of iodine is beneficial in certain types of goiter and acts as a prophylactic in growing children, as shown by Marine (7), disagreement still exists as to the best form or manner in which it should be given. The results obtained in the experiments here reported suggest the possibility of iodized milk for this purpose.

On the basis of 5 parts of iodine in 10,000,000, the amount found to be present in milk when cows were fed 0.1 gm. of potassium iodide, a daily consumption of 1 pint of milk would mean an intake of approximately 0.25 mg. of iodine. While this amount is less than that now recommended by Marine it could easily be increased within limits, by increasing milk consumption or by feeding the cow more iodine. Special feeds containing sea products, particularly fish meal and kelp, have been found to put considerable iodine into milk. Three samples of milk from herds so fed were found to contain 9 parts of iodine in 10,000,000.

The normal milk used in this work contained an abnormally low amount of iodine as compared with that in samples of normal milk produced in other sections. However, this emphasizes the need that may exist for iodine in dairy rations where there is known to be an iodine deficiency in the soil.

Iodizing the general milk supply would insure a constant source of iodine to all persons using milk. It would automatically treat a great many individuals who either through ignorance or lack of funds have had no knowledge of a thyroid deficiency. It would serve as a prophylactic, particularly in young growing children, when an iodine deficiency has not manifested itself in any symp-

tom ordinarily used in diagnosis. Furthermore, the iodine would be furnished in a stable, convenient, and pleasant form.

On the other hand, in cases where a constant supply of iodine would be irritating rather than beneficial, some source of ordinary or non-iodized milk would need to be available. It is true that putting iodine into milk through the cow is rather wasteful as only 10 to 15 per cent of the iodine fed appears in the milk, but by such a procedure the cow is also benefited.

It has recently been claimed by Devereux (8) that the addition of colloidal iodine to milk directly is simpler and can be more accurately controlled than by feeding iodine to the cow. This proposed procedure would require a physician's supervision and hence its usefulness would not be as universal as would an iodized general milk supply. In the last analysis, however, a final decision as to the therapeutic value of an iodized milk supply must rest with the physician and be based upon clinical evidence.

In addition to being of therapeutic value, iodized milk must retain all the beneficial attributes of ordinary milk in order to serve in its fundamental capacity as a universal food. That this is the case has been intimated by Maurer (9). Data on the effect of iodized milk on the rate of growth of young animals is being accumulated by the authors and will be presented in a subsequent report.

SUMMARY.

Data are presented on the weight and iodine content of thyroid glands of white rats fed iodine at different levels, the iodine being supplied as potassium iodide and iodized milk.

Iodized milk was compared with normal milk, and with normal milk plus different amounts of potassium iodide. Prophylactic and curative methods were employed.

In general, smaller thyroid glands containing a greater percentage of iodine resulted whenever iodized milk or potassium iodide was fed than when normal milk or a high calcium-low phosphorus basal grain mixture was used.

The advantages and disadvantages of an iodized milk supply are briefly discussed.

CONCLUSION.

The iodine in iodized milk exerts as great an influence on the size and iodine content of the thyroid gland of rats as does an equivalent amount of iodine administered directly or added to normal milk.

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EXPLANATION OF PLATE 1.

Fig. 1. Thyroid gland of rat on stock diet.

Fig. 2. Thyroid gland of rat on a rickets-producing basal ration for 4 weeks.

Fig. 3. Thyroid gland of a rat on a basal ration for 8 weeks, ultra-violet light during the last 4 weeks.

Fig. 4. Thyroid gland of a rat on a basal ration for 4 weeks, then plus ultra-violet light and 0.000025 gm. of iodine as KI daily.

Fig. 5. Thyroid gland of a rat on a basal ration for 4 weeks, then 50 cc. of iodized milk daily.

Fig. 6. Thyroid gland of a rat on a basal ration for 4 weeks, then 50 cc. of normal milk daily,

1

2

3

4   5   6

(Krauss and Monroe: Thyroid gland iodine cont

A STUDY OF THE ANTIMONY TRICHLORIDE COLOR REACTION FOR VITAMIN A.

IV. THE SOURCE OF VITAMIN B COMPLEX IN THE BIOLOGICAL ASSAY OF VITAMIN A AND THE STABILITY OF VITAMIN A AND OF THE CHROMOGENIC SUBSTANCE IN VARIOUS DILUTING OILS.

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If the color produced by the action of antimony trichloride on cod liver oil be due to vitamin A, we expected it would be a simple matter to compare the colorimetric and biological units, after having found that the blue color produced by the action of antimony trichloride on the cholesterol-free unsaponifiable substance of cod liver oil is a linear function of the concentration, and observed that the dilution curve of cod liver oil approaches a linear function in very dilute solution, which linear function may be considered to be the tangent to the dilution curve at the origin. However some difficulty was experienced in obtaining satisfactory and consistent results with the biological assay.

That the biological assay of vitamin A by the rat growth method has not proved to be entirely satisfactory is shown by the large number of papers that have appeared giving contradictory results, and describing irregularities in the method. A résumé and analysis of some of the work were given by Hume and Smith (1) in 1928, when they came to the conclusion that vitamin A determinations had best be made without a depletion period. However as the amount of vitamin stored in the body of the animal is a variable and unknown factor even when the stock animals are all on a uniform diet, it was considered best for the present work first to deplete the reserves of vitamin A. Hume and Smith particularly emphasize the flattening of the growth curve after 3 or 4 weeks on the experimental diet with those rats from which
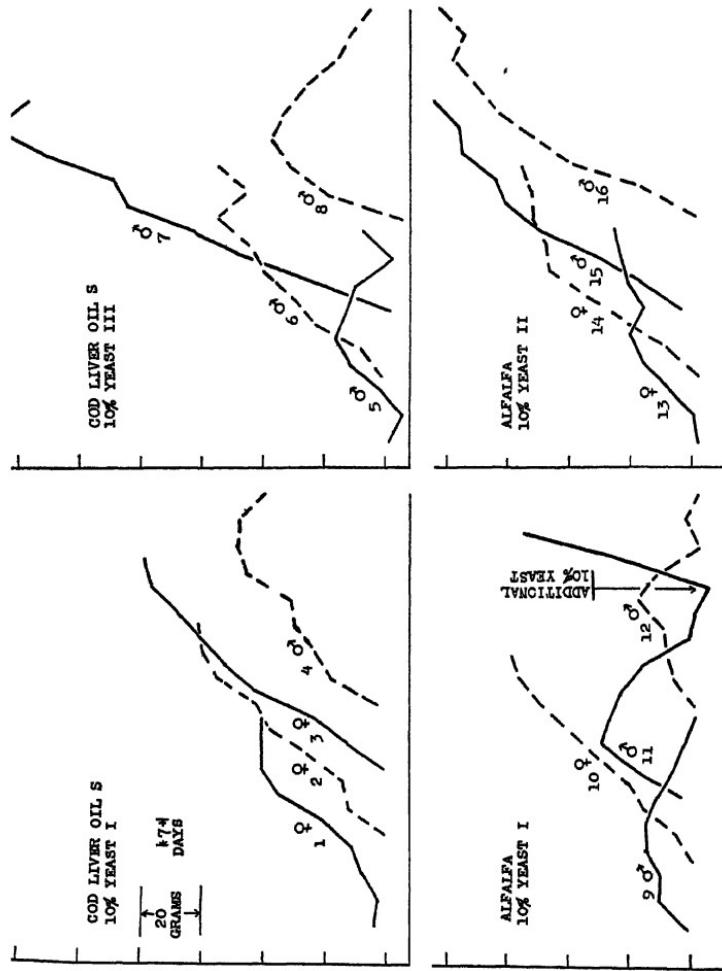
Fig. 1. Growth curves of rats on basal diets containing different samples of yeast.   Rat 1 received 0.0016 gm. per day; Rat 2, 0.0048 gm. per day; Rat 3, 0.0065 gm. per day; Rat 4, 0.01 gm. per day; Rat 5, 0.00072 gm. per day; Rat 6, 0.0062 gm. per day; Rat 7, 0.052 gm. per day; Rat 8, 0.207 gm. per day; Rat 9 and 13 received 0.005 gm. per day of Cod Liver Oil S.   Rats 9 and 13 received 0.005 gm. per day; Rats 10 and 14, 0.01 gm. per day; Rats 11 and 15, 0.03 gm. per day; and Rats 12 and 16, 0.05 gm. per day of dried alfalfa.

the stores of vitamin A of the body had been depleted. Similar flattening of the curve has been reported by many workers. Schmidt-Nielsen (2) show a flattening which they attribute to increased requirement of vitamin A during puberty. Drummond and Morton (3) in discussing the length of the experimental period state that, "In any case the test must not be too prolonged, for even on doses producing normal or nearly normal growth in the early stages for a time, the response may later show a failure which is not made good by subsequently increasing the amount of the supplement." It has been our experience as previously reported (4) that on relatively high dosage of some cod liver oils a flattening of the growth curve was found, which was attributed to a toxicity of the oil, and was not corrected by increasing the source of vitamin A but disappeared on increasing the source of the vitamin B complex. The symptoms of extreme cases of toxicity were those of deficiency of the heat-labile, antineuritic factor or vitamin B. In using different lots of dried yeast, great differences were found in the apparent vitamin content as shown by the premature flattening of the growth curves with sources of vitamin A which should have been sufficient. Increasing the source of vitamin A did not improve the growth, while increasing the source of vitamin B complex did increase the growth as shown by feeding experiments with Cod Liver Oil S in Fig. 1. Fig. 1 gives the curves obtained by feeding various amounts of Cod Liver Oil S to series of rats on two vitamin A-free diets, differing only in the yeast used. In the first, yeast of Lot I, and in the second, yeast of Lot III was used. Both lots of yeast were from the same company and especially prepared for nutritional work. The basal diet consisted of 65 per cent corn-starch, 20 per cent alcohol-extracted casein, 10 per cent dried yeast, 4 per cent Osborne and Mendel salt mixture, and 1 per cent sodium chloride. On the diet with 10 per cent of Yeast I there was an increase in growth with increasing cod liver oil up to 0.006 gm. per day, where the rate of growth was still slightly below normal, but on increasing the source of vitamin A to 0.01 gm. of oil per day a flattening of the curve is obtained after 3 or 4 weeks. With Yeast III continued improvement in growth is obtained up to 0.05 gm. of oil per day where normal growth is observed. However, when the source of vitamin A is increased to 0.207 gm. per

day a flattening and fall of the curve is obtained after 3 weeks, showing the toxicity effect described in a previous paper. On a diet containing 18 per cent of Yeast III normal growth was obtained with 0.207 gm. of oil per day. Hume and Smith (1) describe the flattening of growth curves of rats fed spinach as the source of vitamin A. In preliminary feeding experiments on two samples of yeast we ran series of tests using dried alfalfa as the source of vitamin A (Fig. 1). With Yeast I (the same as used above) on 0.005 gm. of alfalfa per day symptoms of vitamin A deficiency were observed; on increasing the dosage to 0.01 gm. increased growth was obtained. At 0.03 gm. per day normal growth was obtained for 2 weeks followed by a flattening and drop in the curve. After the 8 week period, an additional 10 per cent of yeast was added to the diet with the immediate increase in weight as shown in the diagram. On 0.05 gm. of alfalfa per day weakness, paralysis of the posterior extremities, and convulsions were observed during the 8th week. On Yeast II the flattening of the growth curves was not observed. These results are similar to those reported in a previous paper on feeding small doses of toxic substances as isoamylamine and choline over an extended period of time. What toxic substance is present in the alfalfa used and is counteracted by yeast has not yet been determined. But it shows the extreme variations which might occur in different lots of apparently the same yeast, and emphasizes the fact that simply feeding a definite amount of yeast, as 10 per cent of the diet, does not always insure ample supply of the factors of the vitamin B complex, nor give comparable results. From the symptoms obtained it would seem that dried yeast varies most in the heat-labile factor, vitamin B. If the source of vitamin B complex is sufficiently great, a premature flattening of the growth curve in testing for vitamin A after a period of depletion was not observed, but if the diet contains some substance having a physiological action, such as traces of isoamylamine or choline, not sufficiently balanced by the source of vitamin B complex, the animal may seem to be normal for a few weeks before the effects become apparent. Near the critical point of balance between the source of vitamin B complex and the source of toxic substance, considerable individual variation is often observed, partly due to differences of appetite and food intake.

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In testing a potent cod liver oil for vitamin A the oil must be diluted for accuracy of measurement. It is common practice to dilute the cod liver oil with some natural oil free from vitamin A as peanut oil or olive oil. This practice has been criticized, but any more satisfactory diluent has not yet been found. One of the principal difficulties is the possible destruction of the easily oxidizable substance, vitamin A. Mattill (5), in 1927, points out that in the biological assay of vitamin A the presence of oxidizing catalysts and antioxidizers must be recognized, as the oxidative changes accompanying rancidity in unsaturated animal fats tend to destroy vitamins A and E. The oxidation is retarded in the presence of OH groups. And Willimott and Wokes (6) claim that olive oil should not be used for diluting cod liver oil because of possible presence of organic peroxides, which gradually destroy the vitamin A. Huston, Lightbody, and Ball (7) studied the stabilizing effect of hydroquinone and found it effective in concentrations of from 0.002 to 0.05 per cent. Drummond and Morton (3) suggest that arachis oil and other natural oils seem to contain "components having unsuspected physiological activity," and should therefore not be used. They suggest the use of ethyl oleate; however they found that vitamin A is less stable in pure ethyl oleate than in the natural oils.

In attempting to correlate the colorimetric assay with the biological assay of vitamin A, feeding experiments were made with solutions of the non-saponifiable portion of cod liver oil in olive oil and peanut oil. Cod liver oil was saponified and the non-saponifiable fraction extracted with petroleum ether. The solution stored in a refrigerator was found to remain unchanged for over a year. Aliquots were removed for colorimetric determination and transferred to chloroform by evaporating the petroleum ether at a low temperature and pressure in an atmosphere of nitrogen, and dissolving the residue in anhydrous chloroform. Colorimetric determinations made on Extract IV are given in Table I.

For feeding experiments aliquots of the petroleum ether solution were evaporated at low temperature and pressure in an atmosphere of nitrogen, into weighed amounts of olive or peanut oil.

Young albino rats were used from a stock colony, on a diet of

1000 parts of whole wheat, 500 parts of dried whole milk, 75 parts of dried yeast, 20 parts of iodized salt, supplemented with fresh green lettuce. The animals were weaned at 28 to 29 days of age and placed on the vitamin A-free diet described above and containing 10 per cent of Yeast III. When the stored vitamin A had been depleted, the solution was fed as counted drops from calibrated stalagmometers. An 8 week experimental period was used. Fig. 2 shows the differences in growth response obtained, when the substance was dissolved in olive oil and in peanut oil and also when the olive oil solution had been allowed to stand in a refrigerator for 8 weeks before feeding. To determine whether the difference obtained in growth when fed the solution in olive

TABLE I.

Blue Color Produced by Antimony Trichloride Reaction with Different Concentrations of Extract IV of the Non-Saponifiable Substance of Cod Liver Oil, at Various Times.

Values are given in Lovibond blue units observed at 30 seconds.

Date.	Concentration of solution.			
	100 per cent.	50 per cent.	25 per cent.	12.5 per cent.
Jan. 22, 1929	8.1	3.9	1.9	0.9
Mar. 13, 1929	8.7	4.5	2.5	1.3
" 26, 1929	9.6	4.7	2.3	1.1
Feb. 24, 1930	8.5	4.1	2.3	1.1
Average	8.7	4.3	2.2	1.1

oil and the solution in peanut oil was due to greater destruction in peanut oil or to the presence of some unknown substances in the oil having a deleterious effect, the stability of the chromogenic substance when dissolved in various oils was determined.

A cod liver oil was saponified and the non-saponifiable fraction extracted with ethylene chloride. The ethylene chloride solution was evaporated to dryness in an atmosphere of nitrogen under reduced pressure. The residue was dissolved in petroleum ether and dried with sodium sulfate. Aliquots of the solution were transferred and made up to the same concentration in chloroform solution, in olive oil, in peanut oil, and in coconut oil. Dilution curves were determined at once and again after allowing the
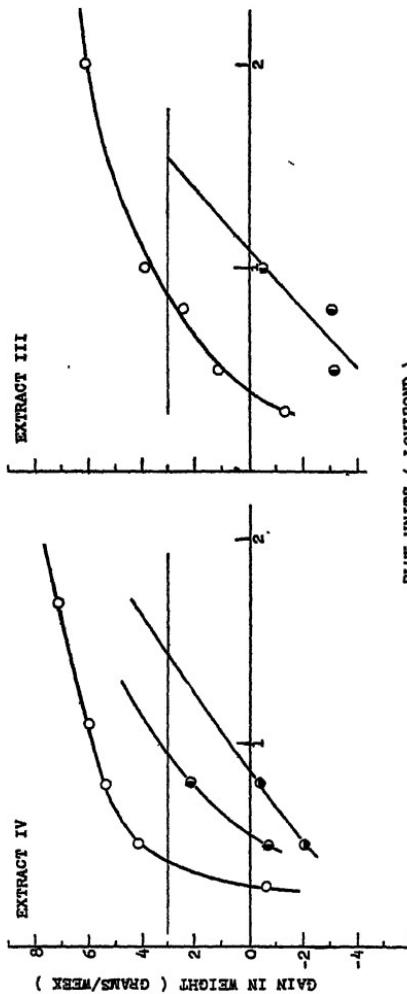
FIG. 2. Growth response of rats fed extracts of the non-saponifiable substance of cod liver oil dissolved in olive oil and peanut oil. Unshaded circles represent values for a solution in olive oil; shaded in the left half the same olive oil solution after standing 8 weeks; lower half shaded a solution in peanut oil.

solutions to remain stoppered 6 weeks in a refrigerator in the dark and cold. The results are shown in Fig. 3. The upper left corner shows that in the chloroform solution the non-saponifi-
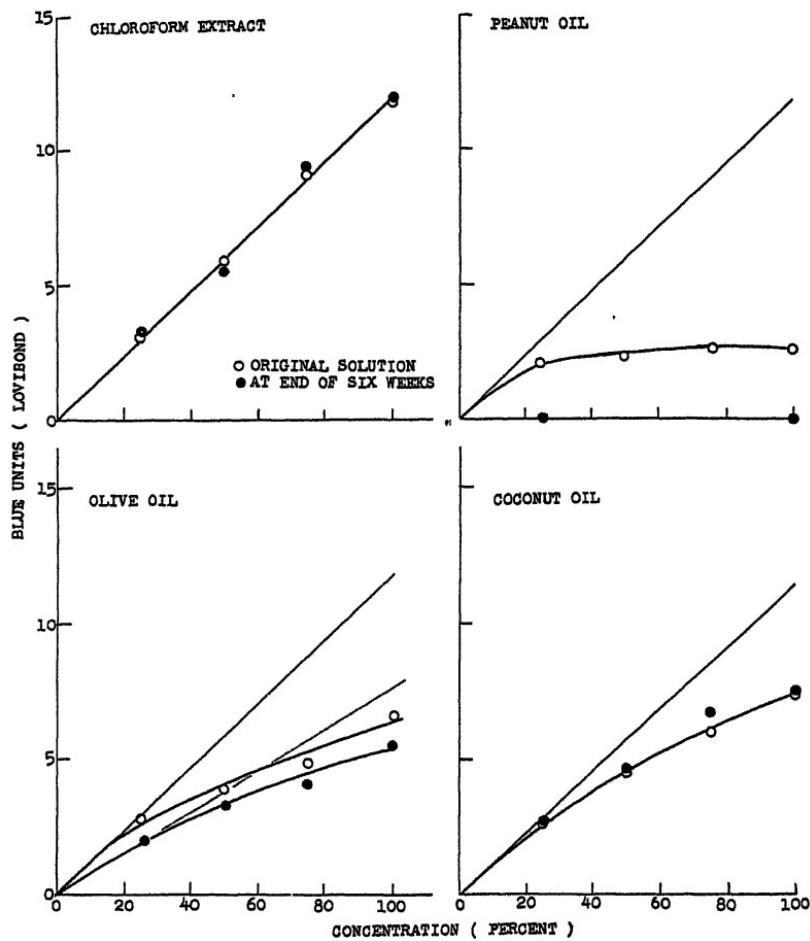
FIG. 3. The stability of the chromogenic substance of cod liver oil when dissolved in olive oil, in peanut oil, and in coconut oil.

able substance produced a color which was a linear function of the concentration and also that it did not vary after being stored for 6 weeks. In each of the other three parts of the diagram are

given the dilution curves of the same concentration of non-saponifiable substance dissolved in peanut oil, olive oil, and coconut oil respectively. The curve representing the linear function produced in chloroform solution is drawn in each section and proves to be the tangent to the dilution curve at the origin for each solution, which was known to contain the same amount of the chromogenic substance. The curves serve to further emphasize how impossible it would be to make a comparison

TABLE II.

Constants for Oils Used as Diluents.

	Iodine No. (Hanus).	Saponification No.	Acid No.
Peanut oil........................	92	200	4.89
Olive "	88	198	0.88
Coconut "	7	253	12.3

TABLE III.

Stability of Chromogenic Substance in Presence of 0.05 Per Cent Hydroquinone.

Concentration.	Diluent.	Blue units observed.	
		When prepared.	After 6 wks.
per cent			
100	Peanut oil.	3.2	2.8
50	" "	2.3	2.3
100	Olive "	6.5	5.3
50	" "	3.9	3.2
100	Coconut "	7.9	
50	" "	3.9	3.7

of the concentration of the chromogenic substance by comparing the color produced by a single concentration of oil giving above 5 blue units. The deviation of the original dilution curve from a linear function is different for each oil, as also was the red color produced during the determination. At 30 seconds after mixing, the observed red units for the 100 per cent solutions were for the chloroform solution 0, for the peanut oil solution 7.7, for the olive oil solution 3.0, and for the coconut oil solution 0.2. Constants for these samples of oils are given in Table II.

There seems to be a rough correlation between the unsaturation, as determined by the iodine number, and the deviation from a linear function; and possibly also between unsaturation and stability of the chromogenic substance.   At the end of 6 weeks the chromogenic substance had been entirely destroyed in the peanut oil, and approximately 30 per cent destroyed in olive oil. There was no destruction in coconut oil.   Consequently animals being fed weighed amounts of solutions of the chromogenic substance in peanut and olive oil are being fed a gradually diminishing source of vitamin A, and this accounts for the feeding results obtained and reported above.   Similar results were obtained with solutions of cod liver oil in peanut and olive oil.   Different samples of coconut oil were tested and found not to be uniform, some samples giving considerable destruction of the chromogenic substance.   The coconut oil also has the disadvantage of being a solid when cooled.

As hydroquinone has been suggested as a possible antioxidant to prevent the destruction of vitamin A, parallel experiments were made with 0.05 per cent of added hydroquinone.   The results are given in Table III.   Hydroquinone prevented destruction with peanut oil but the destruction was not entirely prevented in the solution in olive oil.

SUMMARY.

1. The premature flattening of the growth curve on a vitamin A-free diet supplemented by ample source of vitamin A may be prevented by incorporating sufficient vitamin B complex in the diet.

2. The stability of the chromogenic substance when dissolved in peanut oil, olive oil, and coconut oil was measured.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

XIX. CONCERNING THE COMPOSITION OF THE PHOSPHATIDE FRACTION ISOLATED FROM THE BOVINE TYPE OF TUBERCLE BACILLI.*

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INTRODUCTION.

In an earlier paper (1) we have described the extraction and separation of the lipoids from the bovine tubercle bacilli and reported on the preliminary analysis of the phosphatide fractions. The material designated as Bovine Phosphatide II (1) has now been subjected to an approximate analysis and an attempt has been made to determine the cleavage products which are liberated on hydrolysis.

As was indicated in the former publication (1) experiments by Sabin, Doan, and Forkner[1] have shown that the substance causes in normal rabbits a reaction of specific epithelioid cells and epithelioid or Langhans' giant cells which leads to the formation of tubercular tissue. It has been reported by Doan (2) that the bovine phosphatide acts as an antigen when introduced intravenously in rabbits, and when tested against the serum of tuberculous cattle an aqueous suspension of the phosphatide is precipitated in high dilutions.

The biological properties mentioned above are very similar to the reactions that have been observed by the same investigators in regard to the phosphatides obtained from the human and avian tubercle bacilli. In view of the similarity in biological reactions

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[1] The results of these experiments will be published separately in the near future by the authors named above.

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one would expect to find an analogous composition and indeed in a general way we have found that the cleavage products of all three phosphatides resemble each other. The fatty acids obtained from the avian and bovine phosphatides are almost identical.

The bovine phosphatide, however, contains in the aqueous portion of the hydrolysate a peculiar mucilaginous constituent the like of which we have not encountered in either the human or the avian phosphatides. Another point of difference observed in regard to the bovine phosphatide was its great resistance to hydrolysis when boiled with dilute aqueous acid. In fact we concluded from the results of some preliminary experiments that it would be impossible to effect complete hydrolysis by this procedure. The use of alcohol containing 2 per cent of hydrochloric acid was more successful but it was necessary to boil the substance for many hours with the acid alcohol before complete disintegration took place.

After the phosphatide had been hydrolyzed it yielded about 58 per cent of ether-soluble constituents; the balance was soluble in dilute alcohol or in water. The cleavage products were separated by methods similar to those employed in the analysis of the human (3) and avian (4) phosphatides.

The ether-soluble constituents consisted of fatty acids contaminated with a small amount of a wax-like material but the latter could be removed by reason of its very slight solubility in alcohol. The fatty acids were separated by means of the lead soap-ether treatment and they were found to consist of almost equal parts of liquid fatty acids and a solid saturated fatty acid which corresponded in properties to palmitic acid. The liquid fatty acids had a very low iodine number thus indicating the presence of a large proportion of a saturated liquid fatty acid. The mixture of liquid acids was subjected to catalytic reduction with platinum oxide (5) and hydrogen and the reduction product was removed by repeating the lead soap-ether treatment. The reduced acid was found to be identical with stearic acid and it is probable therefore that the unsaturated fatty acid was oleic acid. The fatty acid isolated from the ether-soluble lead soap, after the catalytic reduction, was an optically inactive oily liquid at room temperature which corresponded in properties and in composition to tuberculostearic acid (6).

The water-soluble constituents could not be separated completely because one of the cleavage products formed a mucilaginous colloidal solution in water. Since this substance could neither be isolated nor removed from the solution by any means at our command it interfered in all operations and it caused excessive foaming when the solutions were concentrated by distillation. In spite of the presence of this peculiar material we were able to isolate a crystalline phenylhydrazine derivative and a colorless crystalline substance from the aqueous solution. These products have been identified as mannose phenylhydrazone and inactive inosite respectively and they will be more fully described in a subsequent paper. Finally we were able to separate a water-soluble barium salt of an organic phosphoric acid which apparently corresponds to barium glycerophosphate.

The phosphatide contained 1 per cent of nitrogen but it was impossible to isolate any nitrogen-containing compound from the hydrolysate. After removing, as far as possible, the various water-soluble constituents, as mentioned above, the alcoholic extract of the residue gave no precipitate with an alcoholic solution of platinum chloride thus indicating the absence of choline. Attempts to separate picrates or picrolonates were equally unsuccessful. Unfortunately it has been impossible up to the present time to secure any information regarding the nitrogen compounds which are contained in the phosphatides of the tubercle bacilli.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*General Properties of the Phosphatide.*</div>

The phosphatide had been isolated from the alcohol-ether extract of the bovine tubercle bacillus and it had been purified by precipitation from ethereal solution by acetone and by methyl alcohol as has already been described (1). The product formed a fine amorphous powder with a faint pink or straw-colored tinge. It was not as soluble in ether as when freshly isolated but it dissolved completely in a large excess of ether. In chloroform or in benzene it was very easily soluble forming perfectly clear solutions. When the substance was rubbed up with water it formed a colloidal solution which when sufficiently diluted with water became almost clear. The aqueous suspensions were precipitated by the addition of acids or alkali. When the alkaline mixture was heated to boil-

ing it gave a clear solution but when the acidified mixture was boiled the precipitate agglutinated forming a heavy scum which did not disintegrate on prolonged boiling.

The aqueous suspension gave no reduction with Fehling's solution until after it had been boiled for some time with dilute acid. When the substance was heated in a capillary tube it turned yellowish at about 200°, sintered at 202°, and melted between 205–208° to a brown liquid. On further heating the melted substance began to decompose at 210° with slight effervescence and the color turned nearly black. On moist litmus paper the phosphatide showed a faint acid reaction. When dried at 56° *in vacuo* over dehydrite the substance lost 1.18 per cent of its weight and on combustion it left 5.33 per cent of ash. On analysis it was found to contain 1.87 per cent of phosphorus and 1.00 per cent of nitrogen.

Hydrolysis of the Phosphatide.

It was tried at first to hydrolyze the phosphatide with 5 per cent aqueous sulfuric acid but this method was abandoned because even prolonged boiling did not cause complete disintegration. It . was found in preliminary experiments when the phosphatide was refluxed with alcohol containing 2 per cent of hydrochloric acid that practically everything gradually went into solution. It was decided therefore to employ the acid-alcohol method of hydrolysis. . Throughout the various operations precautions were observed to exclude air by the use of carbon dioxide or nitrogen until the unsaturated fatty acid had been reduced. All solvents had been freshly distilled and the alcohol had been distilled over potassium hydroxide.

In the first quantitative experiment 2.0385 gm. of material were refluxed for 6 hours with 100 cc. of alcohol containing 2 per cent of hydrochloric acid. At the end of this time the phosphatide had disintegrated yielding a straw-colored solution which contained only a very few small oily drops on the bottom of the flask. When the solution was allowed to cool the oily drops solidified and a slight amount of a white precipitate separated.

Examination of Ether-Soluble Constituents.

The alcoholic solution was diluted with water, extracted with ether, and the ethereal solution was washed with water until the

hydrochloric acid was removed. During these operations emulsions formed which broke very slowly. The washed ethereal solution was concentrated to dryness and the residue was saponified by boiling with 5 per cent alcoholic potassium hydroxide. The solution was diluted with water, acidified with hydrochloric acid, extracted with ether, and the ethereal solution was washed with water until free from hydrochloric acid. On evaporation of the ether a nearly white solid residue was obtained which after it had been dried *in vacuo* weighed 1.1875 gm. corresponding to 58.25 per cent of the phosphatide. The iodine number of the mixed fatty acids determined by the Hanus method was 9.

Examination of Water-Soluble Constituents.

The aqueous solution, after removal of the ether-soluble material, was concentrated under reduced pressure to a small volume. In order to control excessive foaming it was necessary to add a few drops of amyl alcohol at frequent intervals. The residue was dried in a vacuum desiccator over sulfuric acid and potassium hydroxide. The residue was not completely soluble in water but it dissolved in a mixture of alcohol and water. The dilute alcoholic solution was refluxed for 7.5 hours with 5 per cent sulfuric acid in the hope of hydrolyzing the substance which caused the foaming. After the mixture had stood overnight a slight amount of slimy sediment had separated which was collected on a filter and washed with water. This insoluble substance weighed 0.17 gm. The filtrate was again concentrated *in vacuo* but it foamed as badly as at first. The solution was made up to 50 cc. and analyzed for reducing sugars and nitrogen. Found: reducing sugar, by Fehling's solution, calculated as glucose, 15.6 and 15.1 per cent; nitrogen, by Kjeldahl, 0.69 per cent; nitrogen, as ammonia, by aeration, 0.21 per cent.

The insoluble mucilaginous material, mentioned above, was not soluble in any of the ordinary solvents and it was only partly soluble in dilute alcohol. It was boiled continuously in 5 per cent sulfuric acid containing some alcohol for 2 days. The mixture foamed excessively but the substance did not dissolve. After the solution had cooled the substance was filtered off, washed, and dried but there was hardly any loss in weight. The substance gave a positive test for nitrogen but the xanthoproteic and biuret reactions were negative.

Second Hydrolysis.

In a second experiment 11.28 gm. of the phosphatide were re-fluxed for 10.5 hours with 250 cc. of alcohol containing 2 per cent of hydrochloric acid. One-half of the alcohol was removed by dis-tillation and the residue was diluted with water, extracted with highly purified petroleum ether, and the latter solution was washed with water. Emulsions formed which separated slowly. The aqueous solution and washings were united and examined for water-soluble constituents.

Examination of Ether-Soluble Material.

The petroleum ether extract was concentrated to dryness and the residue was refluxed for 2.5 hours with 5 per cent alcoholic potassium hydroxide. A few oily drops remained undissolved and they solidified on cooling, forming white, wax-like globules. The mixture was diluted with water and extracted with ether when the wax-like matter mentioned above was dissolved leaving a clear aqueous soap solution.

Wax-Like Material.

The ethereal solution was shaken with dilute hydrochloric acid and then with water until the acid was removed. On evaporating the ether a nearly white residue was obtained which after it had been dried weighed 0.55 gm. The substance was dissolved in 50 cc. of equal parts of alcohol and ether and the solution on cooling in ice water deposited a white amorphous precipitate. The latter was filtered off, washed with methyl alcohol, and dried. The white powder weighed 0.25 gm. It softened at 56° and melted at 57–58°. In solubility and properties this substance resembled the unsaponifiable wax obtained from the purified wax of the human tubercle bacillus (7).

The mother liquor was concentrated to dryness and the residue which was soluble in alcohol was later added to the fatty acids.

Examination of Fatty Acids.

The soap solution was acidified with hydrochloric acid and ex-tracted three times with ether. After the ethereal solution had been washed with water the ether was evaporated and the residue was dried *in vacuo*. The crude fatty acids weighed 5.9 gm. and

formed a slightly yellowish solid at room temperature. The total yield of ether-soluble material was therefore 6.45 gm. or 57.18 per cent. The fatty acids were separated by means of the lead soap-ether treatment in the usual manner, yielding 3 gm. of solid saturated acid and 2.9 gm. of liquid acid.

Solid Saturated Fatty Acid.

The solid saturated acid was crystallized once from alcohol, once from methyl alcohol, and twice from acetone. The substance was snow-white and it melted at 61–62°, solidified at 57°, and re-melted at 62°.

Titration: 0.3639 gm. substance, dissolved in neutral alcohol, with phenolphthalein as indicator, required 14.00 cc. of 0.1 N KOH. Found, molecular weight 259; calculated for palmitic acid, $C_{16}H_{32}O_2$, molecular weight 256.

The mother liquors from the crystallizations mentioned above were concentrated and the residue was twice recrystallized from methyl alcohol. A snow-white crystalline product was obtained which melted at 61–62°, solidified at 58°, and remelted at 62°.

The melting point and molecular weight of the solid saturated fatty acid would indicate that it consists of practically pure palmitic acid.

Liquid Fatty Acid.

The liquid fatty acid obtained from the ether-soluble lead soap weighed 2.9 gm. The iodine number, determined by the Hanus method, was 12.1, thus indicating the presence of a large proportion of a liquid saturated fatty acid.

The substance was treated with cold alcohol when a small amount remained insoluble which was filtered off, washed with cold alcohol, and dried. This material, which weighed 0.19 gm., melted at 57–58° and resembled the previously mentioned un-saponifiable wax.

The alcoholic solution of the liquid acid was reduced with platinum oxide and hydrogen in the usual manner and the fatty acids were again separated by means of the lead soap-ether procedure, yielding 0.75 gm. of solid reduced acid and 1.75 gm. of a liquid saturated acid.

Reduced Fatty Acid.

The reduced acid was a snow-white solid and after it had been twice recrystallized from methyl alcohol it melted at 69–70°, solidified at 65° and remelted at 69–70°. There was no depression of the melting point when the substance was mixed with pure stearic acid. Since the unsaturated fatty acid gives stearic acid on reduction it is probable that oleic acid was present.

Liquid Saturated Fatty Acid.

The liquid saturated fatty acid which was isolated from the ether-soluble lead soap after the catalytic reduction was a faintly yellow-ish oil at room temperature. When the oil was cooled in ice water it solidified to a white, crystalline mass. That the acid was satu-rated was shown by the fact that in chloroform solution it did not decolorize a dilute solution of bromine.

The acid was optically inactive: 0.6482 gm. of the acid dis-solved in absolute alcohol and made up to 10 cc. showed no rota-tion in a 1 dm. tube.

Titration: 0.3650 gm. of the acid, dissolved in 50 cc. of neutral alcohol, with phenolphthalein as indicator, required 11.89 cc. of 0.1 N KOH. Found, molecular weight 306.

For analysis the substance was dried in a vacuum desiccator for several days and finally *in vacuo* at 61° over dehydrite.

0.1342 gm. substance: 0.1530 gm. H_2O and 0.3734 gm. CO_2.
Found. C 75.88, H 12.75.

In properties and in composition this liquid saturated fatty acid is similar to tuberculostearic acid and it appears to be identi-cal with the liquid saturated fatty acid isolated from the avian phosphatide. The biological activity of the acid has been studied in Dr. Sabin's laboratory at The Rockefeller Institute. It has been found that the acid stimulates the proliferation of epithelioid cells and epithelioid giant cells but perhaps to a somewhat less extent then the crude phthioic acid which was isolated from the Phosphatide A-3.

Examination of Water-Soluble Material.

The aqueous solution, after the fatty acids had been extracted, was concentrated under reduced pressure but owing to excessive

foaming it was necessary to use a flask with a special neck.   The solution contained a colloidal suspension similar to the mucilaginous material observed in the first hydrolysis but as it could not be removed it was temporarily disregarded.   When the solution had been concentrated to a volume of 25 cc. it was mixed with an excess of phenylhydrazine dissolved in a little alcohol.   A crystalline precipitate began to separate immediately.   After the mixture had stood overnight the crystals were filtered off, washed with water, and dried *in vacuo*.   The slightly yellowish crystalline powder which weighed 0.905 gm. represented crude mannose phenylhydrazone.

The excess of phenylhydrazine was removed from the filtrate by the benzaldehyde method.   The aqueous solution was then concentrated under reduced pressure and finally dried in a vacuum desiccator.   The residue was ground up in a mortar under absolute alcohol until a fine powder was produced.   The insoluble material was filtered off, washed with alcohol, and dried *in vacuo*.   The alcoholic solution was reserved for the isolation of barium glycerophosphate.

The alcohol-insoluble substance probably represented an incompletely hydrolyzed carbohydrate complex because it was found to contain phosphorus and after boiling with dilute acid it reduced Fehling's solution.   Every attempt to crystallize the substance failed.

The material was refluxed for 3 hours with 5 per cent sulfuric acid.   After the solution had cooled the sulfuric acid was removed quantitatively with barium hydroxide and the solution was concentrated under reduced pressure to a small volume. . The solution which was acid in reaction was neutralized with barium hydroxide and a barium salt was precipitated by adding 2 volumes of alcohol.   The barium salt was filtered off, washed with alcohol, and dried.   It weighed 0.12 gm. and was later combined with the barium glycerophosphate which was obtained from the alcoholic solution mentioned above.

The excess of barium in the filtrate was precipitated quantitatively with sulfuric acid.   The barium sulfate was removed and the solution after it had been concentrated to a small volume was mixed with alcohol when colorless prismatic needles began to crystallize out.   After the mixture had stood overnight the crystals

were filtered off and washed with alcohol and ether.   The addition of ether to the filtrate caused a further small amount of needle-shaped crystals to separate which were collected and combined with the first lot.   The crystalline substance was identified as in-active inosite, as will be described in a subsequent paper.   The total amount of crystalline inosite weighed 0.4 gm., which is equal to 3.5 per cent of the phosphatide.

The filtrate from the inosite crystals was concentrated to dry-ness when a syrupy residue was obtained which did not crystallize. The syrup was dissolved in 10 cc. of water and this solution showed a dextrorotation of 0.42°.   The solution was mixed with phenyl-hydrazine hydrochloride and sodium acetate in an attempt to prepare an osazone but it was found that a crystalline phenyl-hydrazone began to separate immediately.   After the mixture had stood overnight, the crystals were filtered off, washed with water, alcohol, and ether, and dried.   The substance weighed 0.24 gm. and it was found to be identical with the first lot of phenyl-hydrazone.   As will be described in more detail in a later paper the substance has been indentified as mannose phenylhydrazone. The total amount of hydrazone obtained was therefore 1.145 gm., corresponding to 0.76 gm. of mannose which is equal to 6.7 per cent of the phosphatide.

After the phenylhydrazone had been filtered off the filtrate was heated on the water bath but no osazone separated.   Apparently, therefore, no other reducing sugar such as glucose was present in the syrup.

Separation of Barium Glycerophosphate.

The alcoholic soluton which was obtained on treating the water-soluble syrup with absolute alcohol was diluted with a little water and it was neutralized by adding a solution of barium hydroxide. A heavy amorphous precipitate was produced which was filtered off, and washed with 75 per cent and with 95 per cent alcohol.   The filtrate was saved and examined for nitrogen compounds.

The barium salt after it had been dried *in vacuo* weighed 2.15 gm. and to it was added the 0.12 gm. of the similar barium salt previously mentioned.   For purification the product was dissolved in 25 cc. of water; the solution was warmed and made slightly alkaline with barium hydroxide.   The insoluble matter was

filtered off, washed with water, and dried.   This fraction weighed 0.4 gm. but as it appeared to consist of inorganic barium phosphate it was discarded.   The water-soluble barium salt was precipitated by adding alcohol and the substance was reprecipitated four times in the same manner.   A snow-white amorphous powder was obtained that weighed 1.05 gm.   For analysis the substance was dried at 105° *in vacuo* over dehydrite.

0.1652 gm. substance: 0.1122 gm. $BaSO_4$ and 0.0521 gm. $Mg_2P_2O_7$.
$C_3H_7O_6PBa + 2H_2O$ (343.4).   Calculated.   Ba 39.99, P 9.03.
Found.   " 39.96, " 8.79.

Although the substance had been dried to constant weight at 105° the analytical results agree with the calculated composition of barium glycerophosphate plus 2 molecules of water.

A similar barium salt with anomalous composition was isolated from the cleavage products of the avian phosphatide.   These barium salts have been designated provisionally as barium glycerophosphate but in view of the uncertainty of purity of amorphous substances it is not improbable that the variations in composition from the calculated values are due to the presence of impurities. On the other hand it is not impossible that the organic phosphoric acids which are liberated from the avian and bovine phosphatides are not glycerophosphoric acid.   Up to the present we have had neither time nor sufficient material for a thorough chemical investigation of these organic phosphoric acid compounds.

Examination for Nitrogen Compounds.

The alcoholic solution, after removal of the barium glycerophosphate, contained nitrogen of which only a small amount was present as ammonia.   Much time was consumed in attempts to isolate some nitrogen-containing compound from this solution but our efforts in this direction were entirely unsuccessful.   No insoluble double salts could be obtained with platinum chloride, mercuric chloride, picric acid, picrolonic acid, or with phosphotungstic acid.

SUMMARY.

1. An approximate analysis has been made of the phosphatide isolated from the bovine tubercle bacillus.

2. After hydrolysis the phosphatide yielded from 57 to 58 per cent of ether-soluble material, and the balance was soluble in water or in dilute alcohol.

3. The ether-soluble constituents contained about 5 per cent of wax-like material, 27 per cent of palmitic acid, 7 per cent of oleic acid determined as stearic acid after catalytic reduction, and 16 per cent of an optically inactive liquid saturated fatty acid similar to tuberculostearic acid, $C_{18}H_{36}O_2$.

4. The water-soluble material contained reducing sugar equivalent to about 15 per cent of glucose. From the aqueous solution could be isolated mannose phenylhydrazone, inosite, and a barium salt of an organic phosphoric acid approximately corresponding to barium glycerophosphate.

5. The aqueous solution contained a peculiar mucilaginous material and nitrogen compounds but no information regarding the nature of these components could be obtained.

6. The biological activity of the phosphatide is associated with the liquid saturated fatty acid.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

XX. THE OCCURRENCE OF MANNOSE AND INOSITE IN THE PHOSPHATIDE FRACTIONS FROM THE HUMAN, AVIAN, AND BOVINE TUBERCLE BACILLI.*†

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INTRODUCTION.

In the second paper of this series (1) we described some of the cleavage products obtained from the Phosphatide A-3 isolated from the human type of tubercle bacilli. It was found that the phosphatide yielded about 67 per cent of fatty acids and about 33 per cent of water-soluble constituents. The investigation of .the latter products was incomplete but it was shown that the aqueous solution contained about 13 per cent of reducing sugars calculated as glucose. When the solution was mixed with phenylhydrazine a crystalline derivative separated at room temperature and after this product had been filtered off, the filtrate on heating with phenylhydrazine hydrochloride and sodium acetate gave a good yield of glucosazone. In addition to the crystalline phenylhydrazine derivative we obtained a small amount of a colorless crystalline substance which was not identified and also an amorphous barium salt which corresponded in composition to barium glycerophosphate.

Recently we have been able to identify the phenylhydrazine derivative as mannose phenylhydrazone (2) and the colorless crystalline substance has been identified as inactive inosite (3). The available data gave no clue as to the quantitative relations of mannose and inosite in the phosphatide hydrolysate but some information on this point has now been obtained. A portion of the original water-soluble constituents was available for analysis, and this material has now been examined and it was found to consist of nearly equal parts of inosite, mannose, and another reducing sugar which was levorotatory and which gave glucosazone on treatment with phenylhydrazine. It is evident, therefore, that the Phosphatide A-3 contains a compound which on hydrolysis yields three carbohydrates; viz., inosite, mannose, and a sugar which appears to be similar to invert sugar.

In the analysis of the phosphatide isolated from the avian tubercle bacilli (4) fractions were isolated from the water-soluble constituents which corresponded entirely in properties to the crude inosite and mannose phenylhydrazone obtained from the Phosphatide A-3. After these products had been purified by crystallization they were found to be identical with inosite and mannose phenylhydrazone (5). It is doubtful, however, whether a reducing sugar other than mannose is present in the avian phosphatide because after removing the mannose by means of phenylhydrazine we obtained only a very small amount of glucosazone from the filtrate.

In a preceding paper dealing with the composition of the phosphatide isolated from the bovine type of tubercle bacilli it was mentioned that fractions could be separated from the water-soluble constituents which were identical with inosite and mannose phenylhydrazone (6).

The aqueous portion of the hydrolysate obtained from the bovine phosphatide contained, in addition to inosite and mannose, a substance which formed a mucilaginous colloidal suspension in water. This substance has not yet been isolated and we have, therefore, no information regarding its composition. We were, however, unable to secure any evidence of the presence of any reducing sugar other than mannose in the bovine phosphatide.

Separation of Mixture of Sugars Obtained on Hydrolyzing Phosphatide A-3.

The mixture which was available for examination consisted of the water-soluble material, after the glycerophosphoric acid had been removed as described in the earlier paper (1), and it had been kept in a desiccator for nearly 3 years. It was originally a syrup but at the time of the examination it was a solid which was partly crystalline. The material, which weighed 2.9 gm., was examined in the following order.

A. Isolation of Mannose Phenylhydrazone.—The substance was dissolved in 25 cc. of water and 1.7 gm. of phenylhydrazine dissolved in 5 cc. of alcohol were added. A crystalline precipitate began to separate almost immediately and after the mixture had stood overnight the crystals were filtered off, washed with water, alcohol, and ether, and dried *in vacuo.* The faintly yellowish hydrazone weighed 1.24 gm. corresponding to 0.82 gm. of mannose.

The substance was dissolved in 100 cc. of hot 60 per cent alcohol, the solution was treated with norit, filtered, and allowed to cool, when large colorless rhombic plates separated slowly. The crystals were filtered off, washed with alcohol and ether, and dried *in vacuo.* The dry crystals weighed 0.6 gm. The powdered substance melted with decomposition at 193–194° and there was no depression of the melting point of a mixture with pure mannose phenylhydrazone.

0.1231 gm. substance: 11.45 cc. N at 23° and 744 mm.

$C_{12}H_{18}O_6N_2$ (270). Calculated. N 10.37. Found. N 10.45.

B. Isolation of Inosite.—The filtrate and aqueous washings, after removal of the mannose phenylhydrazone, were shaken with 1 gm. of benzaldehyde. The yellow precipitate which formed was filtered off after the mixture had stood overnight and it was washed with water. The filtrate was extracted with 5 portions of ether, treated with norit, filtered, and concentrated under reduced pressure, and finally dried to a syrup in a vacuum desiccator. Some crystalline material separated which was washed free of the syrupy mother liquor with cold 50 per cent alcohol. The crude crystalline inosite, after it had been dried *in vacuo,* weighed 0.8 gm.

The substance was recrystallized three times from dilute acetic acid and alcohol and finally from hot water by the addition of alcohol. The colorless prismatic crystals were free from water of crystallization and they weighed 0.47 gm.

The substance gave the Scherer reaction, melted at 224–225°, and there was no depression of the melting point when some of the powdered crystals were mixed with pure anhydrous inactive inosite prepared from phytin.

0.1312 gm. substance: 0.0818 gm. H_2O and 0.1915 gm. CO_2.
$C_6H_{12}O_6$ (180).   Calculated.   C 40.00, H 6.66.
                Found.   " 39.81, " 6.97.

The optical properties of the crystals were examined by Dr. E. J. Roberts of this Laboratory in comparison with inosite prepared from phytin. The two crystalline substances were identical and were described as follows by Dr. Roberts: "The crystals are biaxial negative; $\alpha \doteq 1.535$, $\beta \doteq 1.565$, $\gamma \doteq 1.575$. The crystals have a positive elongation and an extinction angle of from 10–20° to the elongation. The trace of the axial plane lies within 10° of the elongation."

C. Isolation of Glucosazone.—The filtrate and washings, after removal of the crude inosite crystals, were treated with norit, filtered, concentrated under reduced pressure, and the residue was dried in a vacuum desiccator. The thick syrupy residue weighed 1.1 gm. The syrup was dissolved in 20 cc. of water and divided into two equal parts. One portion when tested with Fehling's solution gave a heavy reduction. It was dried to a syrup and reserved. The other portion of the solution was mixed with 1.2 gm. of phenylhydrazine hydrochloride, 1.8 gm. of sodium acetate, and the solution was allowed to stand for some time at room temperature but no hydrazone separated. The solution was then heated in a boiling water bath when yellow needle-shaped crystals began to separate after a few minutes. After the solution had been heated for 1 hour it was allowed to cool and the crystals were filtered off, washed with water, and dried *in vacuo*. The crude glucosazone weighed 0.25. gm. The crystals were dissolved in 50 cc. of 50 per cent alcohol, treated with norit, filtered, and the solution was allowed to cool. The glucosazone separated in delicate yellow needles and, after it had been filtered, washed, and

dried, it weighed 0.13 gm.   The powdered crystals melted with decomposition at 205–206° and there was no depression of the melting point when the substance was mixed with glucosazone prepared from pure glucose.

It is evident from the quantity of glucosazone that was obtained that the original syrup must have contained two hexoses, one being mannose; and it was thought at first that the other, which yielded the glucosazone, was most probably glucose.   An examination of the second fraction of the syrup showed, however, that it did not consist of pure glucose.   When some of the material was heated with hydrochloric acid and resorcinol a bright red coloration was obtained and this color reaction is characteristic of ketoses.

TABLE I.

Carbohydrates Separated from Water-Soluble Syrup Obtained on Hydrolyzing Phosphatide A-3.

The dried syrup weighed 2.9 gm.

Substance.	
	gm.
Mannose phenylhydrazone 1.24 gm., corresponding to mannose..	0.82
Inosite, crude crystals..	0.80
Residual syrup, probably invert sugar......................	1.10
Total..	2.72

Rotation: the dried syrup, 0.4603 gm., was dissolved in water and made up to 25 cc.   In a 2 dm. tube the observed rotation was $-0.38°$, hence $[\alpha]_D^{22} = -10.3°$.

In view of the ketose color reaction and the levorotation it seems probable that the syrup contained a mixture of glucose and fructose similar to invert sugar.

The results of this analysis are summarized in Table I.

Examination of Phenylhydrazine Compound Which Had Been Isolated from Water-Soluble Cleavage Products of the Phosphatide from Avian Tubercle Bacilli.—The crystalline substance had been obtained as described in a former paper (4).   The slightly yellowish crystalline powder which weighed 0.55 gm., was recrystallized from 50 cc. of hot 60 per cent alcohol, yielding 0.35 gm. of color-

less rhombic plates which appeared to be identical with crystals of mannose phenylhydrazone. The melting point of the powdered crystals was determined simultaneously with that of mannose phenylhydrazone and with a mixture of the two substances. All three samples melted with decomposition at 192–193°.

The substance was again recrystallized from 60 per cent alcohol, yielding 0.2 gm. of colorless rhombic plates. The melting point was the same as recorded above.

The crystal form, melting point, and other properties indicate that the substance is mannose phenylhydrazone.

Examination of Colorless Crystals Isolated from Water-Soluble Cleavage Products of the Phosphatide from Avian Tubercle Bacilli.— The crude crystalline substance which weighed 0.45 gm., had been obtained as described in a former paper (4). The substance was recrystallized twice from dilute acetic acid by adding alcohol, and three times from water by adding alcohol. Colorless prismatic crystals, characteristic of inosite, were obtained which weighed 0.35 gm. The substance gave the reaction of Scherer. The powdered crystals melted at 224–225° and there was no depression of the melting point when mixed with pure inactive inosite prepared from phytin. The optical properties of the crystals were identical with those of ordinary inactive inosite.

Identification of Mannose Phenylhydrazone and Inosite from the Bovine Phosphatide.

A. Mannose Phenylhydrazone.—The crude phenylhydrazine derivative which had been isolated as described in a preceding paper (6) weighed 1.145 gm. It was recrystallized from hot 60 per cent alcohol and was obtained in the form of large colorless rhombic plates. The optical properties of the crystals were identical with those of mannose phenylhydrazone. The purified crystals melted with decomposition at 195–196° and a mixture of the material with a sample of pure mannose phenylhydrazone determined at the same time melted at the same temperature.

0.2042 gm. dried substance: 18.8 cc. N at 22° and 756 mm.
$C_{12}H_{18}O_5N_2$ (270). Calculated. N 10.37. Found. N 10.59.

B. Inosite.—The crude crystalline inosite, which weighed 0.4 gm., was recrystallized from water by adding alcohol. The

substance separated in colorless prismatic needles characteristic of inosite. It gave the Scherer reaction, melted at 222–223°, and there was no depression of the melting point when mixed with inactive inosite which had been prepared from phytin. The crystallographic properties were also identical with those of inactive anhydrous inosite. Since all the properties were identical with those of inosite the analysis was omitted.

SUMMARY.

1. The water-soluble carbohydrates which are liberated on hydrolyzing the Phosphatide A-3 from the human tubercle bacilli consist of about equal parts of inosite, mannose, and another reducing sugar which appears to be similar to invert sugar.

2. The avian phosphatide on hydrolysis yields water-soluble products containing inosite and mannose.

3. The aqueous portion of the hydrolysate of the bovine phosphatide contains inosite and mannose.

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THE CHEMICAL STUDY OF BACTERIA.

THE PRESENCE OF d-MANNOSE AND d-ARABINOSE IN A COMPLEX CARBOHYDRATE ISOLATED FROM THE CULTURE MEDIUM AFTER THE GROWTH OF TUBERCLE BACILLI.

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INTRODUCTION.

The highly specific character of certain sugars of bacterial origin—*e.g.*, sugars derived from different types of pneumococci (1)—has aroused a marked interest in the study of the chemical composition and biological properties of this class of substance. Several investigators have already directed attention to the carbohydrate, or carbohydrates, produced by the tubercle bacillus. Zinsser (2) studied the so called "residue antigen" in relation to the tuberculin reaction; polysaccharide combinations have been reported by Panzer (3) and Tamura (4). From an alkaline extract of the bacillus Laidlaw and Dudley (5) isolated and studied a carbohydrate, which gave precipitin reactions in high dilution. Mueller (6) obtained a specific carbohydrate from the media on which tubercle bacilli had been cultured.

Within the past few years a series of analytical studies (7) of the culture media during the growth of various types of acid-fast bacilli has been carried on in this laboratory; these studies included a quantitative estimation of the reducing properties of the protein-free solution, and have provided data which should contribute toward interesting generalizations bearing on bacterial growth. Because of the rôle which specific carbohydrates may play in the pathology of disease, and in line with the investiga-

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* National Tuberculosis Association Research Fellow 1929–30.

tions cited, a study of the carbohydrate substances in the media from cultures of tubercle bacilli has been undertaken.

The carbohydrate to be discussed in the present paper was precipitated as a basic lead complex, and the sugar was obtained by decomposition of the lead salt. Dr. Michael Heidelberger, who very kindly tested the crude preparation, reported a definite precipitin reaction in the dilution of 1:1,500,000, with fairly heavy precipitation at a dilution of 1:100,000.

The partially purified carbohydrate has an almost negligible direct reducing value when analyzed by the Shaffer-Hartmann micro method. Acid hydrolysis liberates reducing sugars equivalent to 65 per cent of the original sample (estimated as glucose). *d*-Mannose and *d*-arabinose have been identified in the hydrolysis mixture and appear to represent perhaps 66 per cent of the reducing substances indicated by analysis. The natural occurrence of *d*-arabinose is very rare (8), despite the wide-spread distribution of the levo form, and must represent an unusual chain of chemical transformations, or else an unusual metabolic economy characterizing this type of organism.

One, or possibly two, other hydrazine derivatives have been obtained in impure form and a very small amount of crystalline inosite has been isolated from the hydrolysis mixture.

The medium used as a source of the carbohydrate was supplied by the H. K. Mulford Company of Glenolden, Pennsylvania, and represented a concentrate from 1844 cultures of tubercle bacilli (Strain H-37) inoculated on Long's synthetic medium. The cultures were incubated for 14 months. In view of the glucose value of the hydrolyzed sample of the crude preparation, a yield of about 74 gm. of carbohydrate was estimated; this represented about 0.04 gm. per culture. Shaffer-Hartmann micro analyses of a diluted sample of medium indicated 0.08 gm. of carbohydrate per culture.

Obviously the preparation under discussion cannot be presented as necessarily typical of the products occurring in the medium during the active growth of the bacilli. However, preliminary tests had shown that this media still contained a complex carbohydrate, and the large volume available promised a reasonable yield. It is interesting to note that during the incubation period, apparently no enzyme had been produced to split the polysac-

charide combination into its components. The isolation of a similar carbohydrate fraction from a medium incubated for the normal period of 6 to 8 weeks is now in progress in the laboratory.

EXPERIMENTAL.

Source of Material.—1844 cultures, representing 368,800 cc. of Long's synthetic medium (9), were inoculated with the human type of tubercle bacillus (Strain H-37). After 14 months incubation the cultures were filtered and washed with a considerable volume of water. The medium was candled, treated with 0.01 per cent of hexylresorcinol, and concentrated to 3200 cc.

Procedure for Isolation of Carbohydrate Fraction.—It proved feasible to handle the above concentrated medium in 300 to 500 cc. units. The solution was first treated with neutral lead acetate till no further precipitate formed. Inasmuch as this precipitate, on decomposition, liberated very little carbohydrate, it has been given no further attention as yet. The supernatant solution was concentrated to as small a volume as possible—perhaps one-half the volume of the sample of media taken—and precipitated with basic lead acetate. The filtrate from the basic lead precipitate was again concentrated and treated with an excess of ammonia. The bulky lead precipitate obtained in the presence of ammonia gave essentially the same product as that obtained from the first basic lead precipitate. The basic lead precipitates were decomposed with hydrogen sulfide and the lead-free solutions concentrated to a syrup *in vacuo.*

Dehydration with absolute alcohol left a white or light yellow powder, which contained 2.2 per cent of nitrogen, 1 per cent of phosphorus, and gave 11 per cent of ash. A glycogen test and the Millon test were negative; the preparation did give a slight, but definite biuret test. No precipitation occurred with picric acid, tungstic acid, phosphotungstic acid, barium hydroxide, or mercuric sulfate. These latter properties, with the exception of precipitation by basic lead acetate, are much in accord with the characteristics of the carbohydrate isolated by Mueller (6).

Partial Purification of the Crude Carbohydrate (Fraction HMc-108).—The carbohydrate Fraction HMc-108 falls in the general unit of the products of cell metabolism—designated as D in the chart for the correlation of the chemical study of tubercle bacilli (10) or designated as MB (11).

The crude preparation (Fraction HMc-108) was given a second treatment with H₂S, if necessary. The product was then roughly fractionated with alcohol. When a 20 per cent aqueous solution of the carbohydrate was precipitated with 1 and with 5 volumes of alcohol, and finally concentrated and dehydrated, the first and last fractions contained less of the carbohydrate, as judged by Shaffer-Hartmann analyses. Following the method described by Heidelberger and Avery (12), the more active fraction was further purified by precipitation with methyl alcohol from acidic and from alkaline solutions. Precipitations were carried out with solutions containing 10 or 15 per cent of carbohydrate because recovery from less concentrated solutions was inadequate. No purification by dialysis was attempted.

The best preparation, analyzed by the Shaffer-Hartmann micro method, showed 1 per cent of reducing sugars before hydrolysis and 65 per cent after hydrolysis. This preparation still contained phosphorus. A micro-Dumas determination gave a value of 1.20 per cent and 1.35 per cent for nitrogen. The ash had been reduced to 3 per cent. Despite treatment with norit, the solution was sufficiently colored to make an exact determination of optical rotation difficult.

$$0.3088 \text{ gm. in } 10 \text{ cc.} \quad l = 1, \ \alpha = +0.99°, \ [\alpha]_D = +32.0°.$$

It was thought that the method of Levene and Mori (13) for the hydrolysis of a protein-carbohydrate complex and the elimination of protein might afford a method of obtaining a nitrogen-free preparation. However, after 5.5 hours refluxing with barium hydroxide and treatment with mercuric sulfate, the product still contained nitrogen.

Hydrolysis of Fraction HMc-108.—The above carbohydrate fraction hydrolyzes rather slowly. When 1.6 N H₂SO₄ is used and the solution is heated on a steam bath, 7 or 8 hours are required before the reducing power of the solution has reached a maximum. More rapid hydrolysis occurs if the solution is refluxed by heating on an oil bath with the bath at approximately 125°. Under the latter conditions with N sulfuric acid 30 minutes heating liberates 30.3 per cent of reducing sugars; 60 minutes, 32.5 per cent; 2 hours, 38.7 per cent; 4.5 hours, 41 per cent; and 6.5 hours shows a falling off to 35 per cent.

Although both methods of hydrolysis gave a maximum Shaffer-Hartmann value of about 41 per cent of reducing sugars, the yield of mannose isolated from the hydrolysis mixture after heating at 125° was only 12.6 per cent as compared with a yield of 19.7 per cent from the solution heated on a steam bath. The *d*-arabinose yields were 7.3 and 6.6 per cent, respectively.

Isolation of d-Mannose as Mannose Phenylhydrazone.—The sample of carbohydrate used for hydrolysis had been precipitated from a 20 per cent aqueous solution with 5 volumes of alcohol but had not been further purified. Hydrolysis liberated 41 per cent of reducing sugars as determined by a Shaffer-Hartmann analysis, and duplicate furfural determinations indicated 19 per cent of pentose. Alcoholic extraction of the furfural phloroglucide decreased the weight only 1.8 per cent.

5 gm. of air-dried Fraction HMc-108 were hydrolyzed with 1.6 N sulfuric acid on a steam bath for 6 hours. After removal of sulfuric acid with barium, the solution was concentrated to a volume of 10 to 15 cc. and treated with an excess of freshly distilled phenylhydrazine. After a few minutes, precipitation occurred and the solution set to a thick paste. The yield of 1.48 gm. of mannose phenylhydrazone corresponds to 0.986 gm. of mannose or 19.7 per cent of the weight of the sample hydrolyzed. These crystals were examined under the petrographic microscope[1] and found to give the same interference figure as that reported for mannose phenylhydrazone from the phosphatide of the tubercle bacillus by Anderson and Renfrew (14). A micro-Dumas determination gave the following results.

$C_{12}H_{18}N_2O_5$. Calculated. N 10.37. Found. N 10.15.
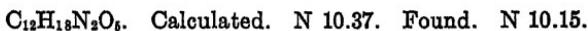
1.82 gm. of mannose phenylhydrazone were decomposed with benzaldehyde. The concentrated mannose syrup was twice dissolved in methyl alcohol and again concentrated. When, even after seeding, crystallization did not occur, the syrup was dehydrated with absolute ethyl alcohol and ether and was dried in a vacuum desiccator. The solution was dextrorotatory.

0.7545 gm. in 10 cc. of H_2O. $l = 1$, α (final) $= +0.97°$. $[\alpha]_D = +12.85°$, $[\alpha]_D$ of *d*-mannose $= +14.25$ ((15) p. 16).

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[1] The examination of these crystals was very kindly made by Dr. E. J. Roberts of this laboratory.

Isolation of d-Arabinose As d-Arabinose Benzylphenylhydrazone.
—The hydrolysate, from which mannose phenylhydrazone had
been removed, was refluxed with benzaldehyde to take up the
excess of phenylhydrazine and to decompose any soluble hydra-
zones. After ether extraction, the solution was concentrated to
a volume of about 5 cc. This concentrate was treated with an
excess of benzylphenylhydrazine and alcohol was added until
the solution cleared. The solution was warmed slightly for a
minute and decanted from any solid precipitated by the addition
of alcohol. Within a few minutes crystals of arabinose benzyl-
phenylhydrazone separated. After several hours the solution
was chilled and filtered. The precipitate was washed with 75
per cent alcohol and then with a large volume of water before the
final washings with alcohol and ether. 0.75 gm. of *d*-arabinose
benzylphenylhydrazone was obtained. About the same yield of
hydrazone was obtained with the use of benzylphenylhydrazine
hydrochloride and sodium acetate. Approximately 10 per cent
of pentose is accounted for as arabinose, whereas furfural de-
terminations indicated 19 per cent.[2] A small amount of a second
benzylphenylhydrazine derivative was separated from the fil-
trate.

After recrystallization from 75 per cent alcohol, the arabinose
hydrazone melted at 172° (uncorrected) and did not lower the
melting point when mixed with an equal quantity of *d*-arabinose
benzylphenylhydrazone prepared from *d*-arabinose (Eastman).
It was found that the melting point of a mixture of the *d* and *l*
forms of the arabinose benzylphenylhydrazone is lowered about
10°. The rate of heating does not greatly affect the melting point
of the mixture; this is of interest in view of the properties of
arabinose diphenylhydrazone, which had already been studied by
Roberts and Anderson (16) in relation to the occurrence of *d*-
arabinose in the wax fraction of the tubercle bacillus. A similar
lowering of the melting point for a mixture of *d*- and *l*-arabinose
p-bromophenylhydrazones has been noted by Fischer (17).

The measurement of optical rotation was made in a solution of
50 per cent (by volume) methyl alcohol and pyridine. An 0.8
per cent solution could be obtained in the alcohol-pyridine mix-
ture, whereas the solubility in methyl alcohol is 0.5 per cent ((15)

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[2] A further quantity of arabinose has been separated from the final fil-
trate as a methylphenylhydrazone.

p. 167), and the alcohol-pyridine solution gave a slightly higher specific rotation. Known preparations of the d and l hydrazones were measured as well as the derivative from the hydrolysis mixture.

l-Arabinose benzylphenylhydrazone. 0.2018 gm. in 25 cc.
$l = 2$, $\alpha = -0.27°$, $[\alpha]_D = -16.7°$.
d-Arabinose benzylphenylhydrazone. 0.2067 gm. in 25 cc.
$l = 2$, $\alpha = +0.30°$, $[\alpha]_D = +18.2°$.
Arabinose benzylphenylhydrazone from the hydrolysis of Fraction HMc-108. 0.2096 gm. in 25 cc. $l = 2$, $\alpha = +0.27°$, $[\alpha]_D = +16.1°$.

A micro-Dumas determination gave the following results.

$C_{18}H_{22}O_4N_2$. Calculated. N 8.48. Found. N 9.07.

The nitrogen analysis suggests the presence of some slight impurity in the benzylphenylhydrazone. Under the microscope the crystals looked homogeneous. The arabinose benzylphenylhydrazone crystals have an extinction angle of about 35°; mannose benzylphenylhydrazone, prepared for purposes of comparison, has almost parallel extinction.

0.8 gm. of arabinose benzylphenylhydrazone was decomposed with benzaldehyde (18). The arabinose, which crystallized directly from the thick syrup, was recrystallized from methyl alcohol and from water. There was no lowering of the melting point when this arabinose was mixed with known d-arabinose. The following determination of optical rotation was made.

0.1348 gm. in 10 cc. $l = 1$, α (initial) $= -1.65°$, $[\alpha]_D$ (initial) $= -122.4°$.
α (final) $= -1.38°$, $[\alpha]_D$ (final) $= -102.4°$.

d-Arabinose has been found in the study of various fractions of the tubercle bacillus (16, 19).

Residue of Fraction HMc-108.—No definite evidence of the presence of uronic acid groupings in the carbohydrate has been obtained. Small quantities of two unidentified hydrazine derivatives have been separated, and a few crystals of inosite have been isolated.

The concentrated neutralized hydrolysate contains a fraction which can be precipitated with alcohol and with basic lead acetate. The product liberated from the basic lead acetate precipitate represented about 16 per cent of the weight of the sample

hydrolyzed; this material had a low direct reducing value which was not increased by further hydrolysis.

In conclusion the writer wishes to express her gratitude to Professor Treat B. Johnson for his helpful advice.

SUMMARY.

1. A carbohydrate fraction has been isolated from synthetic media on which tubercle bacilli (Strain H-37) have been cultured. The crude preparation gives precipitin tests with immune serum.

2. *d*-Mannose and *d*-arabinose have been identified among the products obtained by acid hydrolysis of the carbohydrate.

3. Some properties of *d*-arabinose benzylphenylhydrazone have been noted.

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AN APPARENT EFFECT OF NORMAL VARIATIONS IN THE RESPIRATORY RATE UPON THE EXCRETION OF CHLORIDE AND WATER.

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In 1928, Simpson and Wells (1) discussed the relationship between alveolar carbon dioxide tension and the excretion of water and chloride in the urine. They found that rapid pulmonary ventilation caused an increased output of both urinary constituents, while if an atmosphere of high carbon dioxide tension was breathed a decreased excretion was noted. The evidence tended to show that there was a specific effect upon chloride excretion; *i. e.*, that the changes noted could not be properly attributed wholly to variations in the fluid output.

It has seemed worth while to try to determine whether such effects can be demonstrated in human subjects under ordinary conditions, and indirect data bearing upon this matter are discussed in the present paper. It is obvious that for such a study normal subjects are not suitable, for rather marked variations in chloride excretion and in relative acidity are present which are due, apparently, to the secretion of hydrochloric acid by the stomach. If patients with achlorhydria are used, much of this difficulty is avoided, for the alkaline tide after the meal is absent (2), and the chloride tide is not marked (3). The changes in urinary reaction seen in this condition have been attributed to variations in the rate of pulmonary ventilation by one of the authors (4).

Data have been prepared showing the degree of correlation among the three factors, urinary reaction, water excretion, and chloride excretion in forty tests upon twenty-four patients who were suffering from achlorhydria as demonstrated by the usual

methods of stomach analysis and who were free from renal disease. These patients were studied through morning periods under conditions described elsewhere (5). Chloride was determined by the Volhard-Harvey method. If correlations similar to those predicted from the results of the work of Simpson and Wells can be demonstrated, they will tend to show that under ordinary conditions variations in respiration affect the excretion of water and chloride. Incidentally the presence of such relationships should lend some support to the theory that variations in the respiratory rate are the cause of the variations in urinary reaction seen in these cases.

The method used for analyzing the data was similar to that previously employed (6). Each morning period was considered as a separate unit, and the findings on each specimen in it were compared with those upon every other specimen. The total number of pairs of specimens so chosen was counted, and the number of pairs in which the volume of urine was greater in the more alkaline specimen was also counted. The result was expressed as per cent of the total. To minimize the effect of variations due to other causes the process was repeated disregarding all pairs which did not differ from each other by more than 0.1 pH. The process was then continued, further disregarding pairs of specimens differing from each other by less than 0.5, 1.0, 1.5, and 2.0 pH successively. A similar table was also constructed comparing the rate of sodium chloride excretion and the concentration of sodium chloride in the urine with the urinary reaction.

The results are given in the first half of Table I. In such calculations a figure of 50 per cent is obtained when there is no correlation, 100 per cent represents perfect positive, and 0 per cent complete negative correlation. It is evident that the values of the rates of excretion of both water and salt showed positive correlation with the urinary reaction. The correlation of chloride excretion with the pH values was more nearly perfect than was that of volume with reaction. This better correspondence of the chloride rate tends to confirm the finding of Simpson and Wells that there is a specific effect of some body conditions upon the chloride excretion independent of the effect of the changes upon the volume of fluid passing through the kidneys. There was possibly some degree of negative correlation of chloride

concentration with the urinary reaction, but the relationship was slight and was present only when the differences in reaction were marked.

As controls upon these studies the correlation of each of the factors with the urinary volume is also given. There was no regular increase in alkalinity as the volume increased. Obviously irregularities in the fluid excretion rate due to various causes

TABLE I.

Correlation of Urine Reaction Volume and Chloride Excretion.

Primary variable.	Variation.	Pair No.	Secondary variable.			
			Volume.	Reaction.	NaCl per hr.	NaCl per cc.
			per cent	*per cent*	*per cent*	*per cent*
Reaction.	Any.	452	56		58	51
	Over 0.1 pH.	339	61		63	48
	" 0.4 "	173	71		76	47
	" 0.9 "	88	69		85	50
	" 1.4 "	43	79		90	40
	" 1.9 "	18	83		83	28
Volume.	Any.	527		47	83	42
	Over 5 cc.	459		49	84	38
	" 10 " ·	384		53	84	33
	" 20 "	276		55	83	22
	" 50 "	142		56	76	13
	" 100 "	60		56	65	2

It has been assumed that the values of each dependent variable should vary directly with those of the independent one. The method of selecting the pairs is described in the text.

obscured the positive correlation clearly shown in the first part of Table I. Both chloride excretion and concentration showed changes which paralleled the changes in urinary volume. The first gave a positive and the second a negative correlation as would be expected. Further tables comparing pH values as a secondary variable with the rate of sodium chloride excretion and with the concentration of salt were also prepared. These both showed an absence of any relationship, and have therefore been omitted.

The interpretation of these results seems clear. There was an increase in fluid excretion under conditions leading to the pro-

duction of an alkaline urine. There was also undoubtedly an increased excretion of chloride under the same conditions. This effect upon chloride was distinctly greater than would have been expected from the correlation of volume with reaction and of chloride excretion with volume. Furthermore the chloride concentration showed at most a slight negative correlation when compared with the reaction although the correlation was very marked when the concentration was compared with the volume. These facts imply increase of chloride excretion under the conditions which tend to produce the excretion of an alkaline urine which is not wholly brought about by the increase in volume. Furthermore this effect seems to be fairly specific for the chloride ion; at least similar studies to those given above have shown that there is no such agreement between urinary reaction and phosphate excretion, and Simpson and Wells, in their discussion, conclude that urea is not affected in the same way that sodium chloride is.

CONCLUSION.

These experiments showing a specific parallelism between urinary reaction on the one hand and water and chloride excretion separately on the other suggest that variations in the rate of pulmonary ventilation occurring under ordinary conditions affect the composition of urine, for they are essentially similar to the results obtained in the experiments of Simpson and Wells carried out under extreme conditions.

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QUANTITATIVE ANALYTICAL STUDY OF THE SIMUL-
TANEOUS PRODUCTION OF THIOL ACIDS (R-S-H)
AND SULFONIC ACIDS (R-SO₃-H) FROM DITHIO
ACIDS (R-S-S-R) BY SILVER SULFATE.*

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INTRODUCTION.

Vickery and Leavenworth (7) have recently reported that
cystine could be converted into cysteine by a reaction with silver
sulfate in dilute sulfuric acid solution. Under the conditions
studied, there was obtained a precipitate which contained 70 to
85 per cent of the nitrogen of the original cystine and consisted,
in large part, of a cysteine compound which was considered to be
$(C_3H_5NSO_2Ag)_2 \cdot Ag_2SO_4$. The color reactions of this compound
indicated that it was a silver mercaptide derivative of cysteine.
Cysteine was isolated from the silver derivative by removing the
silver as silver chloride or as silver sulfide. The extensive reduc-
tion that occurred during its formation was accompanied by an
oxidation that resulted in the production of a mixture of sub-
stances, among which ammonia and cysteic acid were identified.

Andrews and Wyman (1) have prepared a compound by the
reaction of mercuric sulfate and cystine which was considered to
be a mercury dicysteine, containing 2 more atoms of mercury.
This hypothesis was supported by experimental observations,
such as the absence of free sulfur from the precipitate obtained
when the mercury compound was treated with hydrogen sulfide,
the dextrorotatory solutions resulting from the precipitation of
l-cystine with mercuric sulfate, and the form of an electrode
potential curve given by the reaction.

The very rapid reduction of cystine to cysteine in dilute silver sulfate solution is particularly interesting in view of the results recently reported by Preisler (5), in which it was shown that cystine and other dithio acids, under somewhat similar conditions, were very difficult to reduce, requiring reversible oxidation-reduction systems of extremely low potential (chromous-chromic or vanadous-vanadic) to cause reduction at a rate rapid enough to measure.

To determine whether this reduction of cystine in silver sulfate solutions was a general reaction for other dithio acids (R-S-S-R) and to obtain information regarding the mechanism of this unusual behavior, the reaction between silver sulfate and various dithio acids was subjected to a detailed study, employing quantitative analytical methods. Dithiodihydracrylic acid, $HOOC \cdot CH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH_2 \cdot COOH$, a compound closely related to the biologically important dithio acid, cystine, has been demonstrated, in aqueous solutions, to follow essentially quantitatively the reaction:

$$3 \; NaOOC \cdot CH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH_2 \cdot COONa + 5 \; Ag_2SO_4 + 3 \; H_2O =$$
$$5 \; AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg + NaOOC \cdot CH_2 \cdot CH_2 \cdot SO_3H + 5 \; NaHSO_4.$$
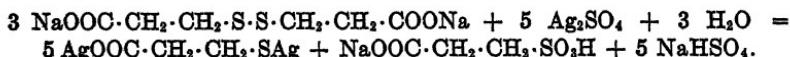
Each product of the reaction has been identified and quantitatively determined, either directly or in the form of characteristic derivatives. The other dithio acids (R-S-S-R) studied, dithiodiglycolic acid, dithiodilactic acid, and cystine, all were found to form some of the corresponding thiol acids (R-S-H) when treated with silver sulfate solutions, but side reactions interfered to such an extent that the same quantitative methods were not applicable. The investigation of the reactions of these compounds is being continued.

Experiments Demonstrating That Reducing Substances Result When R-S-S-R Acids React With Silver Sulfate.

A solution of the dithio acid was prepared by weighing a quantity of the purified acid and dissolving it in hot water or by forming its sodium salt by titrating it with standard sodium hydroxide. When silver sulfate solution was added to the dithio acid solution, a precipitate formed which contained silver in combination. This light yellow precipitate gradually became darker yellow and in

some cases gray or brown. The darker colored products are evidently decomposition products. At no time during the preparation of the solution or the precipitation reaction could the solution containing the dithio acid or its salts be heated directly on a gas flame, even when protected by wire gauze or asbestos, as partial decomposition resulted.

The silver-containing precipitate was filtered off and treated separately or the entire mixture was treated with an excess of potassium iodide and hydrochloric acid. The excess silver in solution and that in the precipitate was thus converted into silver iodide. The reducing substances present were then titrated in the presence of the precipitated silver iodide with potassium iodate and an estimation of the extent of the reduction thereby obtained.

A study was made of the various factors affecting the reaction; concentration of the reactants, temperature, acidity, and length of time.

Dithiodiglycolic Acid.—Light yellow precipitates were obtained which rapidly became darker and finally deep brown. The rate of darkening was very rapid at 90° and moderately rapid at 37°. A strong odor of H_2S was evident on addition of HCl. The amount of reducing substances formed was variable and reached values as high as 115 per cent of the amount calculated for total conversion of R-S-S-R into R-S-H.

Dithiodilactic Acid.—The color changes of the precipitates were similar to those of dithiodiglycolic acid. Some H_2S formed on the addition of HCl. The quantity of reducing substances produced was variable and the rate of production slow, only about 50 per cent being produced before extensive side reactions had taken place.

Cystine.—Gray-yellow to dark gray precipitates were obtained. Some H_2S was formed on the addition of HCl. Precipitation was apparently complete after 15 minutes at 95°. Maximum reducing values of 82 to 90 per cent were obtained when the time allowed for the reaction was sufficiently long. Very long heating resulted in greater H_2S formation and the higher reducing values. Yellow precipitates could be obtained but their reducing values were variable and only from 55 to 75 per cent, depending on the temperature.

Dithiodihydracrylic Acid.—Light yellow and slightly grayish precipitates were obtained. Only slight increases in the color

resulted on prolonged heating at 95°. H_2S formation was doubtful. Precipitation was essentially complete in 10 minutes at 95°. Because more stable precipitates could be obtained with this acid, a more detailed study was made of the conditions necessary for complete reaction. The results (Table I) indicate that a reduction, amounting to about 82.4 to 85.4 per cent, occurred

TABLE I.

Quantity of Reducing Substances Formed When Dithiodihydracrylic Acid Reacts with Silver Sulfate under Various Conditions of Concentration and Temperature.

Experiment No.	Saturated silver sulfate added.	Silver sulfate final normality.	0.1 N dithio acid (Na₂) added.	Temperature of solutions before mixing.	Temperature of digestion.	Time of digestion.	Total volume of mixture.	Reducing substance formed (cc. 0.1 N KIO₃ used).	Fraction of RSSR reduced.
	cc.		cc.	°C.	°C.	min.	cc.		per cent
1	140	0.10	17.99	95	95	10	185	14.90	82.8
2	140	0.10	11.42	95	95	10	175	9.45	82.8
3	100	· 0.10	9.43	95	95	5	160	7.94	84.2
4	100	0.10	10.02	95	95	10	160	8.26	82.5
5	100	0.10	10.01	95	95	10	160	8.25	82.5
6	100	0.10	9.19	95	70	20	160	7.60	82.7
7	100	0.10	12.36	95	70	30	170	10.20	82.5
8	100	0.07	15.53	50	50	5	165	10.58	68.1
9	100	0.07	13.01	50	70	15	160	9.43	72.5
10	100	0.07	12.65	50	70	30	160	10.58	83.6
11	100	0.06	16.49	40	70	30	165	13.72	83.2
12	100	0.05	18.29	30	70	90	170	15.28	83.5
13	100	0.05	14.55	30	70	90	165	11.98	82.4
14	100	0.05	14.31	30	70	150	165	12.12	84.7
15	100	0.05	16.13	30	70	150	170	13.47	85.4
16	100	0.05	15.55	37	37	5	165	9.07	58.3
17	100	0.05	13.69	37	37	100	165	10.80	78.9
18	100	0.05	15.62	37	37	675	165	13.25	84.8

when the solutions were subjected to the proper conditions of temperature and concentration for a sufficient time. Since Vickery and Leavenworth (7) isolated some cysteic acid from the filtrates of their silver-cystine reaction and since the reaction postulated by them for cysteine and cysteic acid formation called for the formation of 83.3 per cent of R-S-H, it seemed probable

that this type of reaction might be occurring essentially quantitatively for dithiodihydracrylic acid. Quantitative analytical experiments were devised to determine the exact nature of the reaction.

Quantitative Analytical Experiments Showing the Nature of the Reaction between Dithiodihydracrylic Acid and Silver Sulfate.

From the data given in Table I and from other preliminary experiments, it seemed that the reaction occurred essentially quantitatively according to the following equation.

$$3 \; NaOOC \cdot CH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH_2 \cdot COONa + 5 \; Ag_2SO_4 + 3 \; H_2O =$$
$$5 \; AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg + NaOOC \cdot CH_2 \cdot CH_2 \cdot SO_3H + 5 \; NaHSO_4.$$
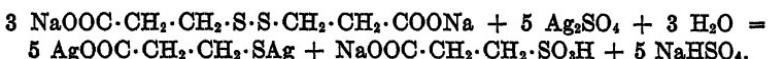
That this reaction takes place quantitatively has been proved by the following.

(1) The calculated weight of $AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg$ was obtained.

(2) When the silver salt obtained in (1) was treated with HCl and KI for the removal of the silver and the resulting mixture titrated with KIO_3, an amount of KIO_3 equivalent to the calculated -SH content was reduced.

(3) After the removal of the silver salt of (1) by filtration the excess silver was removed by the addition of standard NaCl and the AgCl formed filtered off. The filtrate was demonstrated to contain the expected amount of acid ($NaHSO_4$ and RSO_3H) by NaOH titration.

(4) The neutralized solution resulting from (3) contained the theoretical amount of chloride (from the originally known excess) as determined by titration with $AgNO_3$.

(5) The filtrate, after the removal of the silver salt of (1), contained the theoretical amount of sulfate and silver determined by precipitating simultaneously as $BaSO_4$ and AgI and weighing.

(6) The filtrate from (5) was demonstrated to contain the expected amount of acid ($NaHSO_4$ and RSO_3H).

(7) The expected quantity of $HOOC \cdot CH_2 \cdot CH_2 \cdot SO_3H$ was shown to be present in the filtrate from (5) by isolation and weighing as the barium salt.

(8) Microanalyses were made for the constituent elements of the silver compound obtained in (1) and the barium salt of the acid in (7) and the results corresponded to the calculated values.

Similar experiments were carried out with thiohydracrylic acid to show that the reaction given above was peculiar to the dithio acid. For thiohydracrylic acid, the experiments showed that the reaction took place according to the following equation.

$$NaOOC \cdot CH_2 \cdot CH_2 \cdot SH + Ag_2SO_4 = AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg + NaHSO_4.$$
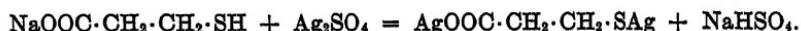
The calculated quantities of substances were obtained from experiments similar to (1–6) above and no sulfonic acid could be demonstrated by the procedure cited in (7). Microanalyses gave the calculated results for the constituent elements of the silver compound of thiohydracrylic acid. The analytical data show that the silver salt obtained from the dithio acid and that from the thiol acid are identical.

<div align="center">EXPERIMENTAL.</div>

Dithiodihydracrylic acid was prepared according to the method of Biilmann (2) which consists of the preparation of the thiol acid by a reaction of β-bromopropionic acid with potassium ethyl xanthate, followed by the decomposition of the resulting complex by ammonium hydroxide, removal of the xanthogenate amide, and liberation of the thiol acid by hydrochloric acid. After extraction with ether and purification by fractional distillation *in vacuo*, the thiol acid was converted into the dithio acid by oxidation with iodine. Purification of the dithio acid was accomplished by dissolving it in dilute sodium hydroxide and precipitating with hydrochloric acid, followed by three recrystallizations from water. The acid so prepared required the theoretical quantity of standard sodium hydroxide for neutralization.

The silver sulfate solution was prepared from silver sulfate crystals which had been washed several times by decantation to remove any reduced silver or organic matter which might react with the silver sulfate at the higher temperatures. · The solution was standardized by titrating with sodium chloride, with potassium chromate as indicator.

A solution of the disodium salt of dithiodihydracrylic acid was made by dissolving 3.1535 gm. of the acid in the amount of sodium hydroxide necessary to bring it to a phenolphthalein end-point, after which it was diluted to 500.00 cc. (Required, 253.6 cc. of 0.1184 N NaOH; theoretical, 253.3 cc.)

Series 1. Experiments 21 through 26.—To 25.00 cc. of the dithio salt solution were added 25 cc. of water and the mixture heated in a beaker immersed in boiling water. 100.00 cc. of 0.04810 N silver sulfate were added after being heated nearly to boiling.

After digestion for a period of 60 to 90 minutes, the precipitate was filtered off and washed in water. The filter paper was then perforated and the precipitate washed into a beaker with water

TABLE II.

Analytical Data for Determination of Products of Reaction between Dithio-dihydracrylic Acid and Silver Sulfate.

Series 1, experiment No.	-SH formed (cc. 0.1000 N KIO₃).	Acid formed (cc. 0.1184 N NaOH).	Residual silver in filtrate (indirect, cc. 0.1288 N AgNO₃).	Series 2, experiment No.	Silver-containing precipitate.	Acid formed (cc. 0.1184 N NaOH).	Residual silver and sulfate in filtrate (AgI + BaSO₄).	RSO₃H as Ba salt.
					gm.		*gm.*	*gm.*
21	12.49	12.48	20.43	31	0.4011	12.65	1.1106	0.0736
22	12.53	12.66	20.70	32	0.3968	12.72	1.1122	0.0726
23	12.55	12.63	20.52	33	0.4008	12.53	1.0828	0.0712
24	12.69	12.59	20.59	34	0.4005	12.58	1.1112	0.0732
25	12.65	·12.61	20.59	35	0.3992	12.63	1.1069	0.0734
26	12.54	12.61	20.47	36	0.4016	12.66	1.1040	0.0710
Average.......	12.57	12.55	20.56		0.4000	12.62	1.1046	0.0725
Theoretical....	12.50	12.67	20.88		0.3999	12.67	1.1038	0.0724
Per cent.......	100.6	99.0	98.7		100.0	99.6	100.1	100.1

followed by a solution of 3 gm. of KI in 25 cc. of water in which solution the precipitate is soluble.

10 cc. of 1 per cent starch solution and 5 cc. of 6 N HCl were added to the mixture which was then titrated with 0.1000 N KIO₃ until the first appearance of a greenish color, caused by the blue starch-iodine color in the presence of the yellow precipitate of AgI formed when the HCl was added. The validity of the iodate titration as an estimation of the R-S-H present is based on the well established methods of Biilmann (2) and of Klason and

Carlson (3). (KIO$_3$ required, average, 12.57 cc.; theoretical, 12.50 cc.; per cent of theoretical, 100.6.)

To the filtrate from the Ag precipitate were added 50.00 cc. of 0.1000 N NaCl and the mixture heated until the AgCl was in proper condition for filtration. This precipitate was filtered off, washed, and rejected. The filtrate was then titrated with 0.1184 N NaOH to a phenolphthalein end-point. The amount of acid formed, as NaHSO$_4$ and RSO$_3$H, was thus determined. (NaOH required, average, 12.55 cc.; theoretical, 12.67 cc.; per cent of theoretical, 99.0.)

This neutral solution was titrated with 0.1288 N AgNO$_3$, K$_2$CrO$_4$ being used as indicator, to determine the excess chloride, from which the silver which remained could be calculated. (AgNO$_3$ required, average, 20.56 cc.; theoretical, 20.88 cc.; per cent of theoretical, 98.7.)

Series 2. Experiments 31 through 36.—The precipitation was carried out in the same manner as in Series 1. The silver salt was filtered off on a sintered glass filtering crucible, washed with alcohol and ether, dried in a desiccator over CaCl$_2$, and weighed. (Average weight, 0.4000 gm.; theoretical weight, 0.3999 gm.; per cent of theoretical, 100.0.)

To precipitate the Ag$^+$ and the SO$_4^=$ as AgI and BaSO$_4$, a solution of 2 gm. of neutral BaI$_2$ in 20 cc. was added to this solution and the mixture digested until the precipitate was in condition for filtering through an asbestos lined Gooch crucible. BaI$_2$ must be used for this precipitation because this salt and other iodides are soluble in alcohol, which was later added, whereas the other Ba salts usually used are not sufficiently soluble. The crucible was heated at 200° for 2 hours and then gently ignited on a Bunsen flame to constant weight. The precipitate was weighed up as AgI + BaSO$_4$. (Average weight, 1.1046 gm.; theoretical weight, 1.1038 gm.; per cent of theoretical, 100.1.)

The filtrate so obtained was titrated with 0.1184 N NaOH and the amount of acid formed as NaHSO$_4$ and RSO$_3$H was thus determined. (NaOH required, average, 12.62 cc.; theoretical, 12.67 cc.; per cent of theoretical, 99.6.)

The solution was then evaporated down to dryness, 10 cc. of water added, and the solution filtered and the paper washed with water. After again evaporating down to about 10 cc., 60 cc. of

95 per cent alcohol were added. The white flocculent precipitate was filtered off on a sintered glass filter and washed with alcohol and ether. Since the rapid precipitation causes the formation of a mixture of anhydrous and hydrated salt, the salts were heated at 180 to 200° to constant weight in order to form the anhydrous salt (Rosenthal (6)) and then weighed as $Ba(-OOC \cdot CH_2CH_2 \cdot SO_3-)$. (Average weight, 0.0725 gm.; theoretical weight, 0.0724 gm.; per cent of theoretical, 100.1.)

Analyses of Precipitates.—The $Ba(-OOC \cdot CH_2 \cdot CH_2 \cdot SO_3-)$ precipitates from the six determinations were dissolved from the crucibles with water and combined. The solution was evaporated to dryness on the steam bath and 30 cc. of water were added and the solution filtered. The filtrate and washings were combined and evaporated to a volume of about 25 cc. and then cooled to 0°. The resulting well crystallized salt, containing 5 H_2O of crystallization, was filtered off on a sintered glass crucible and weighed. To the filtrate was added about 5 times its volume of ethyl alcohol and the resulting precipitate filtered off and heated at 180° to constant weight. Weight of 5 H_2O salt, 0.3803 gm. Weight of anhydrous salt prepared from the filtrate, 0.1127 gm. While no particular attempt was made for a quantitative recovery of the recrystallized product, the total recovery, calculated as anhydrous salt, was 0.4028 gm.; theoretical, 0.4344 gm.; per cent recovery, 92.7.

Microanalyses of the 5 H_2O salt gave the following results. 5.400 mg. of substance gave 1.880 mg. of carbon dioxide and 1.910 mg. of water leaving a residue of 3.265 mg. 7.230 mg. of substance gave 4.485 mg. of barium sulfate by precipitation with barium chloride. 6.590 mg. of substance lost 1.640 mg. on heating at 180°.

Found: C, 9.49 per cent; H, 3.95 per cent; ash, 60.46 per cent; Ba, 36.50 per cent; moisture, 24.88 per cent.

Calculated: C, 9.49 per cent; H, 3.71 per cent; ash ($BaSO_4$), 61.50 per cent; Ba, 36.20 per cent; moisture (5 H_2O), 23.73 per cent.

The silver salt, $AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg$, could not be purified by recrystallization, therefore, the sample was used as prepared. Drying of the sample was accomplished by placing it in a desiccator over calcium chloride. The substance was not stable even at a

TABLE III.

Analytical Data for Determination of Products of Reaction between Thiohydracrylic Acid and Silver Sulfate.

Standardization of thiol acid		Series 1	Na-thio-hydracrylate (cc. 0.1273 N)	-SH formed (cc. 0.1000 N KIO₃)	Acid formed (cc. 0.1273 N NaOH)	Residual silver in filtrate (indirect, cc. 0.1288 N AgNO₃)	Series 2	Na-thio-hydracrylate (cc. 0.1273 N)	Silver-containing precipitate (gm.)	Acid formed (cc. 0.1184 N NaOH)	Residual silver and sulfate in filtrate, Ag + BaSO₄ (gm.)	RSO₃H as Ba salt (gm.)
cc. 0.1273 N NaOH	cc. 0.1000 N KIO₃								*gm.*		*gm.*	*gm.*
11.88	15.49	41	11.84	15.80	12.01	25.22	51	13.89	0.5254	13.95	0.8987	0.0000
11.87	15.32	42	11.89	15.48	11.93	25.30	52	13.97	0.5230	13.83	0.9073	0.0000
11.86	15.42	43	11.90	15.60	11.98	25.29	53	14.00	0.5244	13.85	0.8986	0.0000
11.81	15.40	44	11.80	15.81	11.98	25.60	54	13.90	0.5235	13.86	0.9040	0.0000
		45	11.87	15.68	12.03	25.05	55	13.98	0.5247	13.85	0.9036	0.0000
		46	11.84	15.47	11.96	25.63	56	13.91	0.5252	13.87	0.8912	0.0000
15.41		Average.	11.86	15.64	11.86	25.35		13.94	0.5244	13.87	0.9006	0.0000
15.10		Theoretical.		15.10		24.97			0.5288	13.94	0.8963	0.0000
102.0		Per cent.	(Standard, 100.0)	103.8	101.0	101.5		(Standard, 100.0)	99.2	99.5	100.5	
11.86 (Standard, 100.0)			(Standard, 100.0) 11.86									

temperature of 80° so heating could not be used as a means for drying the sample.

7.905 mg. of substance gave 3.255 mg. of carbon dioxide and 0.990 mg. of water. 7.140 mg. of substance gave 6.315 mg. of silver chloride after decomposition by nitric acid and precipitation by hydrochloric acid.

Found: C, 11.22 per cent; H, 1.40 per cent; Ag, 66.56 per cent.
Calculated: C, 11.26 per cent; H, 1.26 per cent; Ag, 67.46 per cent.

The silver salt, $AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg$, prepared by the reaction of thiohydracrylic acid, $HOOC \cdot CH_2 \cdot CH_2 \cdot SH$, with silver sulfate gave on microanalysis the following results: C, 11.41 per cent; H, 1.41 per cent; Ag, 66.40 per cent; which indicate that it is identical to the silver salt prepared by the reaction of dithiodihydracrylic acid with silver sulfate.

Thiohydracrylic Acid Series.—The purification of thiohydracrylic acid entailed considerable difficulty. After three fractional distillations *in vacuo* of the material prepared by the method described under the preparation of dithiodihydracrylic acid, the preparation showed an -SH titer of about 115 per cent of the acid titer. To obtain a purer sample, since the dithio acid could be prepared in very pure condition, the thiohydracrylic acid was made by reducing the purified dithio acid by zinc and hydrochloric acid. The thiol acid was then extracted from the mixture by ether and water removed from the ether solution by Na_2SO_4. After removal of the ether by distillation, the thiohydracrylic acid was fractionally distilled *in vacuo*. The ratio of the -SH titer to the acid titer of all fractions was then only 1.02. Further attempts at purification by vacuum distillation did not produce a better sample. It was, therefore, necessary to use the acid prepared as described. Whether the failure of the agreement between the -SH value and the acid value was due to impurities or an overtitration of the -SH value because of the difficult endpoint, has not been established.

The same experimental procedures as described in Series 1 and Series 2 of dithiodihydracrylic acid were carried out for thiohydracrylic acid except in the case of the initial precipitation which was made by slowly adding the thiohydracrylic acid sodium salt to the silver sulfate solution. If the silver sulfate solution

was rapidly added to the salt solution, the amount of silver contained in the precipitate was too low, possibly due to precipitation of a monosilver salt into which the second silver substituted only slowly.

Table III demonstrates the agreement between the experimental and the calculated values and indicates that the previously given equation for the reaction is essentially correct.

Microanalyses of the silver salt, $AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg$, were made on the sample prepared as described. Drying the sample was accomplished by placing it in a desiccator over calcium chloride.

8.300 mg. of substance gave 3.475 mg. of carbon dioxide and 1.050 mg. of water. 10.845 mg. of substance gave 9.570 mg. of silver chloride after decomposition by nitric acid and precipitation by hydrochloric acid.

Found: C, 11.41 per cent; H, 1.41 per cent; Ag, 66.40 per cent.
Calculated: C, 11.26 per cent; H, 1.26 per cent; Ag, 67.46 per cent.

DISCUSSION.

The experimental procedure, which analyzes for the end-products of the reaction, cannot be expected to yield much information regarding the mechanism by which the final result is obtained. However, a few suggestions as to the possible course of the reaction seem appropriate here.

The reaction might be due to an intramolecular oxidation and reduction in which the -S-S- grouping is in part oxidized to $-SO_3H$ and in part reduced to -SH, as might be expressed by the following equation.

$$HOOC \cdot CH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH_2 \cdot COOH + 3\,H_2O = 5\,HOOC \cdot CH_2 \cdot CH_2 \cdot SH \\ +\ HOOC \cdot CH_2 \cdot CH_2 \cdot SO_3H.$$
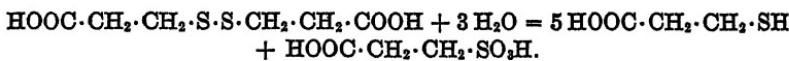
The silver ion, in this case, might serve the purpose of removing one of the products of the reaction as the insoluble silver salt, $AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg$, thereby shifting a possible equilibrium and causing the reaction to go essentially to completion. Any other chemical process which would remove one of the products written on the right of the equation or increase the reactants written on the left would tend to increase the formation of sulfonic acid. Thus oxidation of R-S-H by iodine should yield

R-SO$_3$H in large quantity through a cycle of reactions by way of R-S-S-R and the above reaction. However, iodine rapidly oxidizes R-S-H only to R-S-S-R. The existence of a true equilibrium is therefore to be doubted for the reaction given in the above equation.

That the reaction might be due to a peroxidation by means of the silver or resulting products of the reaction is another possibility. Assuming that 2 Ag add to the -S-S- grouping, forming a critical complex which decomposes into 2 AgOOC·CH$_2$·CH$_2$·SAg, a free SO$_4$ group would be available as an oxidizing agent. This SO$_4$, combining with H$_2$SO$_4$ or an alkali metal sulfate, would yield the corresponding persulfate, H$_2$S$_2$O$_8$. Persulfates react with silver salts to form Ag$_2$S$_2$O$_8$ which decomposes into silver peroxide, Ag$_2$O$_2$, which is reported to be a powerful oxidizing agent for organic substances (4).` Thus when 3 mols of R-S-S-R react with 3 Ag$_2$SO$_4$, there would be formed 6 AgOOC·CH$_2$·CH$_2$·SAg and 3 SO$_4$ would be liberated. 6 oxidizing equivalents would then be available directly or through the persulfate or silver peroxide for the oxidation of one of the -SH groups formed to a sulfonic acid. Experimental work to test these hypotheses is being carried on.

The formation of R-S-H from R-S-S-R by the addition of silver sulfate, which is without pronounced oxidizing or reducing action, is indeed astonishing in view of the recent work reported by Preisler (5) in which it was shown that the reduction of dithiodihydracrylic acid required reversible oxidation-reduction reagents of very low potential such as chromous chloride (E_0 = -0.400 volts). With such reagents, the reaction proceeded at a measurable rate and required many hours before essentially complete reduction was accomplished, whereas, with silver sulfate the reaction is finished in a few minutes.

Since it has been shown that under favorable conditions R-S-S-R may react to produce the corresponding R-S-H compounds, R-S-S-R compounds must now be considered as being capable of acting as reducing agents of the intensity of the R-S-H compounds, instead of only being considered as oxidized compounds difficult to reduce and relatively inert chemically.

Because of the close relationship between dithiodihydracrylic acid and cystine, the information obtained regarding the silver

derivative of thiohydracrylic acid obtained from the former may be of some assistance for establishing the constitution of the silver derivative of cysteine obtained from cystine. Vickery and Leavenworth (7) assigned to their cysteine silver compound the formula, $(C_3H_5NSO_2Ag)_2 \cdot Ag_2SO_4$, and for those cases in which the sulfate content was lower than the calculated value, they assumed that their compound was a mixture, containing some $(C_3H_5NSO_2Ag)_2 \cdot Ag_2O$. Since no sulfate enters into combination with the thiohydracrylic acid derivative and the silver replaces the hydrogens of the carboxyl and thiol groups, by analogy the formula of the cysteine silver compound might be supposed to be $AgOOC \cdot CHNH_2 \cdot CH_2SAg$. When sulfuric acid combined with this compound, as it might do in solutions of proper acidity, it might readily combine with the amino group to form the amine salt, $(AgOOC \cdot CHNH_3 \cdot CH_2SAg)_2SO_4$.

The biological importance of the reaction becomes apparent when it is recalled that R-S-S-R and R-S-H compounds are widely distributed in biological material, as cystine, cysteine, glutathione, and reduced glutathione. Taurine, $H_2N \cdot CH_2 \cdot CH_2 \cdot SO_3H$, is also found either free or combined as a normal constituent of certain body fluids, and possibly arises from the decarboxylation of cysteic acid, $HOOC \cdot H_2NCH \cdot CH_2 \cdot SO_3H$. Since the initial reactants and probable derivatives of the end-products are present in the animal body, it is possible that this reaction may play an important rôle in the sulfur metabolism of the body through the agency of the many catalysts which are known to be present. Investigation of this reaction is being extended to include glutathione and other biologically important sulfur compounds.

SUMMARY.

1. Reducing substances, capable of being oxidized by iodine, result when dithiodiglycolic acid, dithiodilactic acid, cystine, or dithiodihydracrylic acid are allowed to react with silver sulfate solutions.

2. Quantitative analytical experiments have shown that for dithiodihydracrylic acid the reaction occurs essentially quantitatively according to the following equation.

$$3 \ NaOOC \cdot CH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH_2 \cdot COONa + 5 \ Ag_2SO_4 + 3 \ H_2O =$$
$$5 \ AgOOC \cdot CH_2 \cdot CH_2 \cdot SAg + NaOOC \cdot CH_2 \cdot CH_2 \cdot SO_3H + 5 \ NaHSO_4.$$
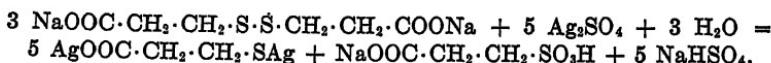
3. The chemical and biological significance of the reaction is discussed.

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THE SOURCE OF EXCESS CALCIUM IN HYPERCALCEMIA INDUCED BY IRRADIATED ERGOSTEROL.*

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Numerous investigators have shown that large doses of irradiated ergosterol produce a hypercalcemia. The source of this excess of serum calcium has not been determined, but several workers believe that it is the body tissues especially the bones and not the ingested calcium of the food. Light, Miller, and Frey (1), Hess, Weinstock, and Rivkin (2), and Brown and Shohl (3) claim that very large amounts of vitamin D cause a decrease in the percentage of bone ash. Gil (4) believes that the calcium comes from the non-osteoid tissues. This theory is hardly tenable in view of the fact that many of the internal organs become calcified under the influence of large amounts of irradiated ergosterol. The most direct proof that the source of calcium is the body tissues and not the food is furnished by the work of Hess, Weinstock, and Rivkin (2, 5). They have demonstrated that it is possible to produce a vitamin D hypercalcemia on a diet low in calcium. This would seem to rule out the food as an important source of the additional calcium. However, Harris (6) and Bills and Wirick (7) have shown that the toxicity of irradiated ergosterol can be intensified by increasing the amount of calcium in the food. Harris believes that in "rickets the primary disturbance is apparently a lowered retention of phosphate or calcium from the gut."

The experiments of Hess and associates are subject to criticism in that the diet used may have contained sufficient calcium to produce a hypercalcemia although it was low in respect to this element. On the assumption that a 10 kilo dog has 800 cc. of

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blood, 500 cc. of which are serum, it would require but 25 mg. of excess calcium to raise the level from 11 mg. to 16 mg. per 100 cc. of serum. The amount of calcium that a dog normally excretes by way of the urine is small. Greenwald and Gross (8) in numerous experiments have found that at most it is only a few mg. per day. If the antirachitic factor acts by increasing absorption of calcium from the intestine or decreasing its excretion into the intestine, there would need be but a small amount in the food to supply sufficient calcium to produce a marked hypercalcemia in the course of a few weeks. In the case of a 100 gm. rat an increased retention of about 0.5 mg. of calcium would be sufficient to produce a marked hypercalcemia. In some of their experiments, Hess and coworkers fed to rats approximately 0.8 mg. of calcium per animal per day. This is more calcium than is normally present in the serum of a 100 gm. rat.

In the following experiments a diet practically free of calcium has been used. The effect of administration of large doses of irradiated ergosterol in conjunction with this diet has been compared with the effect of equal doses of ergosterol given in addition to a diet containing calcium.

EXPERIMENTAL.

Dogs were used as experimental animals. The preparation of a diet which is calcium-free but complete in every other respect is a difficult task. It is almost impossible to prepare a calcium-free protein in the quantity necessary for dog feeding experiments. However, since these experiments were to be of short duration it was thought that the omission of protein from the diet would have little, if any, influence on the results. After various modifications the calcium-free diet used was composed as follows:

	per cent
Lactose	43
Lard	22
Sucrose	33
Cane-sugar charcoal	2

To 160 gm. of the above was added the alcoholic extract of 25 gm. of wheat embryo.

Obviously, this diet is both incomplete and unbalanced, but we

believe that in an experiment of this nature only the following conditions need be fulfilled: (*a*) The diet must contain sufficient vitamin B (old terminology) to prevent loss of appetite. (*b*) It must supply sufficient energy to prevent excessive destruction of body tissue. This is of prime importance in minimizing the liberation of calcium which undoubtedly results from the breakdown of body tissue. (*c*) Lastly, it should contain adequate roughage to insure normal intestinal movements and evacuation.

The ash of 10 gm. of this diet gave no qualitative test for calcium by the oxalate method. The caloric content of this diet is almost identical with that of the modified Karr-Cowgill diet previously described (9) and which was used with several of the controls. The amount given per kilo of body weight was the same as that for the Karr-Cowgill diet; *viz.*, 16 gm. per kilo. As stated in an earlier publication (9) dogs invariably lose their appetites after several days of excessive irradiated ergosterol administration. That this diet was well taken by the animals until the irradiated ergosterol had time to produce its characteristic anorexia is shown by the following typical protocol which is also similar to the protocols obtained on the Karr-Cowgill diet.

Dog 83.—Male, weight 10 kilos. Feb. 11, 1930, began feeding 160 gm. of Ca-free diet; Feb. 12–17, eating 160 gm. of diet daily; Feb. 16, feces soft and black; Feb. 18, began giving 1 mg. of irradiated ergosterol per kilo per day; Feb. 18–Mar. 8, eating full amount of ration daily; Mar. 9, stopped eating; Mar. 10–20, consumed no food; Mar. 11, discontinued ergosterol and gave 20 gm. of Ca gluconate per os; Mar. 21–28, ate irregularly; Mar. 28, experiment discontinued.

With the exception of rather soft feces there were no indications of gastrointestinal disturbance due to the high carbohydrate content of the diet. The feces during this period were too soft to be formed but were not watery.

The calcium-free diet was fed until the color of the feces showed that the charcoal was being excreted for 3 or 4 days before beginning the administration of ergosterol. This precaution was taken to insure the elimination of any calcium remaining in the gut from the previous diet. The ration of several of the control animals was the same as previously reported (9) and contained about 0.25 per cent calcium. The irradiated ergosterol used was a preparation containing 10 mg. of activated material per cc. of

solution, and according to the manufacturers had an antirachitic potency of 1500 D.[1]

Some of the controls reported here, Chart I, were used in connection with another experiment, and for the most part were given 1 cc. of the ergosterol preparation daily regardless of the size of the animal. However, as most of the dogs weighed slightly over 10 kilos the amount given was somewhat less than 0.1 cc. per kilo of body weight. One large dog (Dog 69) which weighed 17.5 kilos was given 2 cc. of the preparation daily for 6 days, and one of the dogs (Dog 76) which received 1 cc. of the preparation per day weighed but 9 kilos. The daily dose of these two animals was thus slightly more than 0.1 cc. per kilo. Two of the controls (Dogs 75 and 82) were given 0.1 cc. per kilo per day, which was the same as that given to all but one of the experimental animals.

As seen from Chart I there was a marked increase in the concentration of serum calcium in every one of these controls. In all cases except Dogs 64 and 82 the calcium of the serum was more than 15 mg. per 100 cc. within 2 weeks after the beginning of ergosterol administration. The highest concentration attained was 20 mg. per cent (Dog 76). The administration of ergosterol to Dog 64 was discontinued after 2 weeks although the concentration of serum calcium had increased only a little more than 2 mg. Before using Dog 82 as a control it had previously been given the calcium-free diet plus ergosterol. After this first experimental period several weeks elapsed before the vitamin D preparation was again administered. During the interval the animal was fed the regular daily allowance of the standard basal diet. This ration was also given during the second ergosterol period. As seen from Chart I there was a rather marked rise in calcium concentration to 14 mg. per 100 cc. of serum within 2 weeks. For 2 additional weeks the concentration remained about constant although the ergosterol feeding was continued and the dog consumed its food readily. After approximately 4 weeks of this treatment the administration of ergosterol was discontinued, and whole milk powder (Klim) suspended in water was given instead

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[1] The irradiated ergosterol was kindly furnished by the Winthrop Chemical Company, Inc. of New York. The designation 1500 D has the usual meaning that this sample of irradiated ergosterol had 1500 times the vitamin D potency of standard cod liver oil.
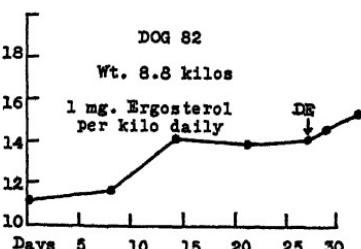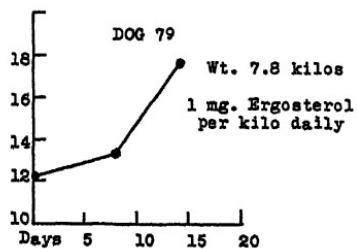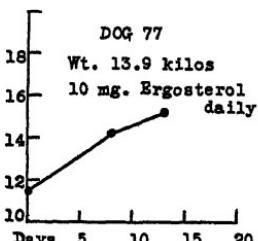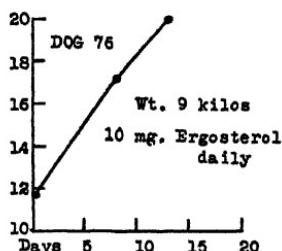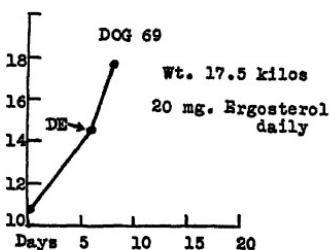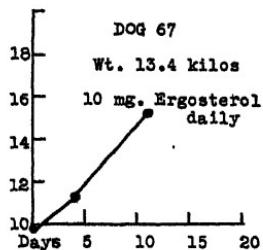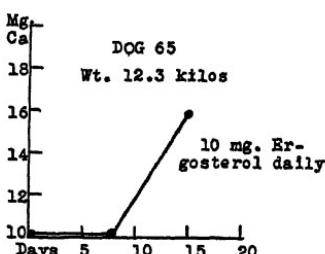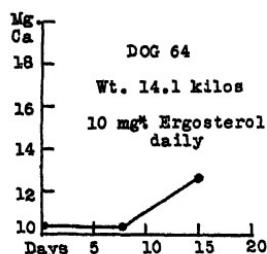
CHART I. The effect of irradiated ergosterol on the concentration of the serum calcium of dogs receiving a diet containing 0.25 per cent of calcium. *1 cc. of oil solution (1500 D) contains 10 mg. of ergosterol; DE, discontinued ergosterol.

of the basal ration.   The change in diet was followed by a slight increase in concentration of serum calcium.   As will be seen from later experiments the effect of ergosterol lasts for some time after the administration of it is discontinued.   The increase in serum calcium in this case must have been due to either the greater intake of calcium or to a greater availability of the calcium.

Besides the results reported in Chart I, two dogs were each given 1 cc. of the ergosterol solution per day, but the sera of which were not analyzed for calcium before beginning the administration of the vitamin D preparation.   The dogs weighed 16.7 and 12.2 kilos respectively and showed calcium concentrations of 17.6 mg.
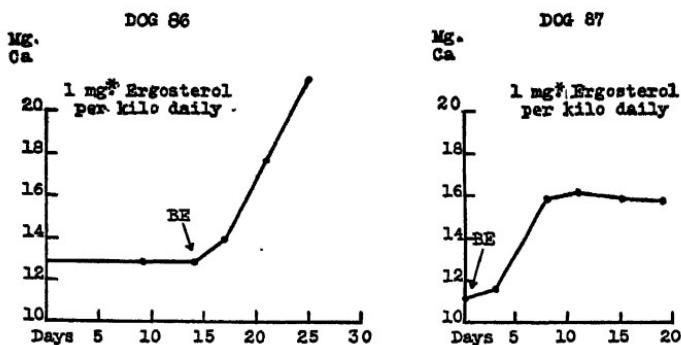
CHART II.   The effect of irradiated ergosterol on the concentration of the serum calcium of dogs receiving the calcium-free diet to which has been added 1 per cent of calcium as calcium gluconate.   * 1 cc. of oil solution (1500 D) contains 10 mg. of ergosterol; BE, began administration of ergosterol.

and 16.8 mg. per 100 cc. of serum after 2 weeks of ergosterol administration.

In addition to the controls mentioned above, two dogs were given the calcium-free diet to which 1 per cent of calcium in the form of calcium gluconate had been added.   As seen from Chart II a daily dose of 0.1 cc. of irradiated ergosterol solution per kilo of body weight produced a marked hypercalcemia in each of these animals in less than 2 weeks.   Dog 86 (Chart II) received this high calcium diet for 2 weeks before the administration of ergosterol was begun.   The concentration of serum calcium remained constant during this period, but immediately increased when ir-

radiated ergosterol was administered. This experiment shows that the high blood calcium obtained with these two dogs was not due alone to the high calcium content of the diet, but that irradiated ergosterol was also necessary to produce this condition. Furthermore, the experiments with these two dogs show that the results obtained with this abnormal diet to which calcium has been added are identical with the results obtained with the balanced Karr-Cowgill diet.

Of the above twelve control dogs which were fed approximately 0.1 cc. of ergosterol solution per kilo per day for 2 weeks, ten showed a concentration of serum calcium in excess of 15 mg. per 100 cc. of serum. The other two developed a hypercalcemia within the same length of time but to a less extent.

The results obtained when irradiated ergosterol was fed to dogs receiving the calcium-free diet are given in Chart III. 0.1 cc. of ergosterol solution per kilo per day was given to each of these dogs except Dog 78 which was given a total of 1 cc. per day.

Chart III shows that with the exception of Dog 81 there was but a very slight increase in the concentration of serum calcium, although the ergosterol was given for approximately 3 weeks as compared to 2 weeks for the controls. At the end of this period the administration of the ergosterol was discontinued and either calcium gluconate or food containing calcium was given. Dog 78 was fed Klim and yeast daily for 6 days and Dog 85 received Klim for 3 days. All of the others except Dog 81 were given 200 cc. of a 10 per cent solution of calcium gluconate by stomach tube. As shown by Chart III there was a marked and prompt rise in the concentration of serum calcium in every case when calcium was supplied in the diet. Blood samples were taken from the dogs receiving calcium gluconate at the time this salt was given and just 20 hours later. There was an average increase of 3.12 mg. of calcium per 100 cc. of serum during this short time. Equal quantities of calcium gluconate given to dogs which had received no ergosterol produced no increase in concentration of serum calcium at the end of the same period of time.

Shortly after the beginning of ergosterol administration Dog 81 developed a severe distemper which may or may not explain the hypercalcemia in this animal. However, since it takes such a small amount of calcium to produce a definite hypercalcemia, it
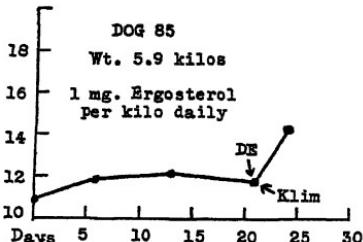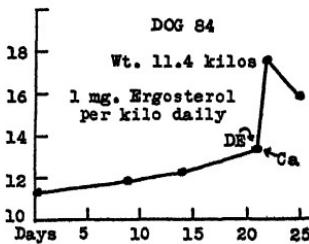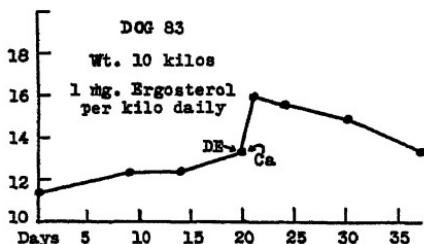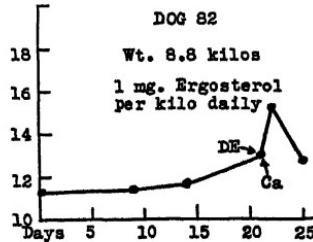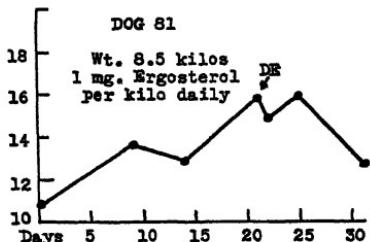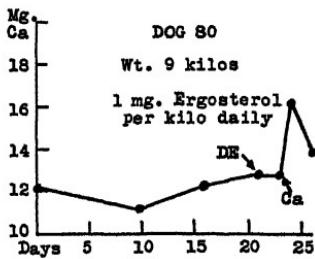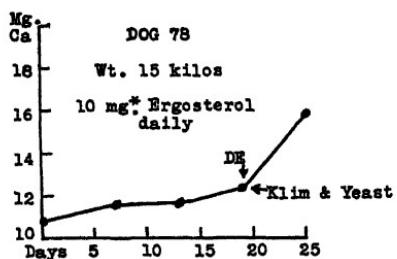
CHART III. The effect of irradiated ergosterol on the concentration of the serum calcium of dogs receiving a calcium-free diet.   * 1 cc. of oil solution (1500 D) contains 10 mg. of ergosterol; DE, discontinued ergosterol; Ca, administered 20 gm. of calcium gluconate by stomach tube; Klim, whole milk powder.

is possible that there was a sufficient amount of calcium left in the intestinal tract to produce the results obtained. This may also account for the slight increase in concentration of calcium in the serum of the other dogs on the calcium-free diet, or there may have been traces of calcium in the diet although as previously stated the ash from 10 gm. of the ration gave no precipitate with oxalate.

In addition to the above another dog (Dog 88) was given the calcium-free diet for 21 days. During the last 16 days of this period the animal received 0.1 cc. of the ergosterol preparation per kilo per day. At the beginning of ergosterol administration the calcium of the blood serum was 11.5 mg. per 100 cc. and 16 days later it was 11.4 mg. This experiment was discontinued at this point.

It is apparent from the above data that the excess calcium of irradiated ergosterol hypercalcemia comes from the intestinal tract as suggested by Harris. Whether the results are due to an increased absorption or to a decreased elimination has not been determined.   .

SUMMARY AND CONCLUSION.

Dogs given approximately 0.1 cc. of a solution of irradiated ergosterol (1500 D) per kilo of body weight per day invariably develop a pronounced hypercalcemia within 2 weeks if there is sufficient calcium in the diet.   .

If the diet fed is calcium-free, dogs receiving a daily dose of irradiated ergosterol equal to that of the controls show only a slight increase in concentration of serum calcium in 3 weeks. If calcium is added to the diet at the end of this period there is a sudden and pronounced increase in serum calcium concentration.

These data indicate that the source of the excess of calcium in irradiated ergosterol hypercalcemia is the food and not the body tissue.   .

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SOME OBSERVATIONS ON THE BEHAVIOR OF VITAMIN A IN OR FROM PRIMARY SOURCES.*

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With materials known to be potent sources of vitamin A, studies have recently been made to determine the following: (*a*) the effect of storage for considerable periods of time on the vitamin A content of dry plant tissue; (*b*) the effect of the presence of a rancid fat on the vitamin A of plant tissue during periods of storage; (*c*) whether the vitamin A of such different types of plant material as fruit, leafy plant tissue, and root vegetable is soluble in a vegetable oil; (*d*) the effect of ultra-violet light on vitamin A after its extraction from plant tissue.

The Sherman and Munsell (1) and Sherman and Burtis (2) quantitative method for determining vitamin A has been employed in each of these experiments, the results of which are briefly presented under the various headings given below.

Effect of Storage for Considerable Periods of Time on Vitamin A Content of Dry Plant Tissue.

Earlier work (3, 4) has tended to show that the vitamin A in plant tissue is relatively stable at least to certain treatments which involve but short periods of time. During the course of some studies in which dry spinach was being used for a source of vitamin A the question arose as to whether the vitamin content remained fairly constant under the conditions of storage that the sample was subjected to in the laboratory at that time.

It was proposed to test the spinach for vitamin A and to repeat the tests on the same sample at intervals of 6, 12, and 15 months. The material which was very finely ground was kept throughout

the storage period in a Mason jar on the laboratory shelf exposed to the diffused light from a nearby window and, except at the time of making the tests, the jar containing the sample was kept tightly sealed.

With the growth and survival of experimental animals fed the test samples as criteria, the potency of the spinach at various periods of storage may be judged from the results shown in Table I.

Apparently there was a loss in vitamin A potency of the spinach during the 15 months storage. Whereas at the beginning of the storage period 20 mg. of the material daily supplied sufficient vitamin to allow the experimental animals to make a definite gain in weight during the 8 weeks experimental period, 30 mg. of the sample daily 6 months later furnished an insufficient amount

TABLE I.

Growth and Survival of Experimental Animals Fed Test Samples of Spinach.

Stage of storage.	Daily portions of sample fed.	No. of animals.	Average gain in weight.	Survival period.
	gm.		*gm.*	*days*
Beginning of storage period.......	0.020	5	17	56
After 6 mos.....................	0.030	2	−22	56
" 12 "	0.050	2	12	55
" 15 "	0.100	2	24	56
Negative controls................	0.000	3	−8	36

of the factor to maintain the weight of the animals. After the lapse of 1 year 50 mg. daily gave an average gain in weight slightly below and at the end of 15 months 100 mg. daily resulted in a gain slightly above that obtained with 20 mg. at the beginning of the experiment.

Although the number of animals used is probably too small to evaluate quantitatively the amount of destruction of the vitamin that took place, it would seem quite justifiable to express this loss in at least approximate terms. Considering the amounts of the test material fed and the resulting growth of the experimental animals receiving these portions it is estimated that the destruction of the vitamin during the 12 to 15 months of storage was something in the neighborhood of 70 per cent.

It was observed that the spinach became considerably lighter in color upon standing, this being especially noticeable on the surface of the sample and in those portions immediately next to the sides of the glass container. Just what connection this fading of color had with the destruction of the vitamin was not determined but in view of the results of other experiments now in progress in this laboratory and of work reported later in this paper it is believed that light may have been one of the agencies responsible for both the fading of the color and the destruction of the vitamin.

Effect of Presence of a Rancid Fat upon the Vitamin A of Plant Tissue during Storage.

It has been shown (5, 6) that the vitamin A in secondary sources of this factor is sometimes destroyed by products which are found in rancid fats. To determine whether vitamin A of plant tissue is subject to similar destruction when exposed to a rancid-fat it was planned to make tests for vitamin A of a sample of dry spinach to which a rancid fat was added and to compare these results with those obtained in the experiments where dry spinach alone was used.

Dry powdered spinach and rancid butter fat were well mixed in such proportions as to give a sample containing 20 per cent of the added fat. Observing the same conditions as to storage, as have been described, vitamin A tests were made in parallel with those reported in Table I. Making allowance for the amount of butter fat in the sample the mixture was fed at the same levels as were employed in the first series. That the rancid fat itself contained little or no vitamin A and could have contributed no measurable amount of this factor in the spinach-rancid fat mixture is evident from the fact that animals fed as much as 30 mg. portions of the fat daily made no growth and their survival was about the same as the negative controls or those fed the basal vitamin A-free diet only.

The results of feeding this rancid fat-treated sample of spinach to litter mates of the animals referred to in Table I are given in Table II.

Comparing these data with those in Table I it is seen that the presence of the rancid fat caused a decidedly greater destruction

of vitamin A, during the storage period, than that which took place in the sample to which no fat was added. After 15 months of storage the spinach-rancid butter fat sample contained considerably less than one-fifth of the amount of vitamin A that was present at the start of the storage period.

It is apparent that vitamin A of plant tissue as well as the vitamin A in secondary sources is susceptible to the destructive action of the products of rancid fat. It is possible however that the action is not so pronounced in the former type of material as in the latter for the work of both Fridericia (5) and Powick (6) on this subject indicates that the vitamin A of lard and egg yolk is destroyed very rapidly. Reference to the results of the first 56 day period in the present experiments, however, shows that the

TABLE II.

Results of Feeding Rancid Fat-Treated Sample of Spinach to Litter Mates of Animals of Table I.

Stage of storage.	Daily portions of sample fed.	No. of animals.	Average gain in weight.	Survival period.
	gm.		*gm.*	*days*
Beginning of storage period.......	0.024	5	15	56
After 6 mos......................	0.036	2	−44	47
" 12 "	0.063	2	−13	40
" 15 "	0.125	2	−30	45
Negative controls.................	0.000	3	−8	36

animals fed the spinach-rancid fat mixture made nearly the same total gain in weight during this first 8 weeks as did those receiving the dry spinach alone; thus indicating the absence of any rapid or marked destruction of the vitamin A in the rancid sample during this time.

Solubility of Vitamin A of Plant Tissue in a Vegetable Fat.

Reports have appeared in the literature from time to time which might lead one to believe that vitamin A of plant tissue is not soluble in, or is not extractable by, natural fats or oils. From work of Osborne and Mendel (7) and Day (8), however, it appears that the vitamin A of at least certain types of plant tissue is soluble in vegetable oils.

It was thought possible by the authors of this paper that the type of plant tissue might be a factor which would determine whether or not the vitamin A present would be soluble. For this reason three different types of plant materials including a fruit, a root vegetable, and leafy plant tissue were treated with a vegetable oil and the filtered extracts tested for their vitamin A potency. Tomatoes were selected as the fruit, carrots as the root vegetable, and broccoli as a representative of leafy plant tissue, each of these materials being recognized as a very good source of vitamin A.

The materials were dried in an air oven and each triturated in a mortar with 100 cc. of peanut oil which tests had shown to contain practically no vitamin A. The mass was filtered and the

TABLE III.

Solubility of Vitamin A of Plant Tissue in a Vegetable Fat.

Plant tissue extracted.	Weight of dry plant tissue extracted with 100 cc. peanut oil.	Daily portions of oil extracts fed as supplements to vitamin A-free diet.	No. of animals.	Average gain in weight.	Survival.
	gm.	*gm.*		*gm.*	*days*
Dry tomato pulp........	26	0.150	3	46	56
" carrots.............	26	0.150	3	57	56
" broccoli.............	9	0.150	2	14	56
Negative control........		0.000	1	−31	24
Peanut oil..............		0.150	1	−25	27

clear oil free of suspended matter thus obtained fed to the experimental animals at the levels indicated in Table III.

It appears from these results that the vitamin A of dried plant tissue of the types here investigated is readily soluble in, and extractable by, a vegetable oil.

Effect of Ultra-Violet Light on Vitamin A after Its Extraction from Plant Tissue by Petroleum Ether.

In connection with our investigations of the properties of vitamin A from plant sources we desired to know something regarding the stability of this vitamin when exposed to ultra-violet light. Earlier work (9, 10) has shown that the vitamin A in butter fat and cod liver oil is completely destroyed upon exposure for a

short time to the light from a mercury quartz lamp. Evers (11) has recently stated that, while the vitamin A of cod liver oil is readily destroyed by ultra-violet light, sunlight is still more destructive to the vitamin.

In the present work, instead of irradiating the plant tissue directly, petroleum ether extracts of dried carrots which served as the source of the vitamin were irradiated. Small volumes, 25 to 30 cc., of the colored extracts were placed in 50 cc. quartz flasks, the flasks tightly stoppered, and the samples irradiated at a distance of 12 inches from a mercury quartz lamp. The exposure was continued until practically all color was destroyed; which required from 3 to 5 hours. The colorless extracts were then

TABLE IV.

Ultra-Violet Light Effect on Vitamin A Extracted from Plant Tissue by Petroleum Ether.

Extracts fed.	Amount of starch-evapor-ated extract residue fed daily.	No. of animals.	Total aver-age gain in weight.	Survival.
	gm.		*gm.*	*days*
Non-irradiated extracts........	0.5	4	52	56
Irradiated extracts...........	0.5	4	−38	37
Negative controls............	0.0	2	−34	30

evaporated upon corn-starch and these residues fed to rats receiving a vitamin A-free basal diet. In parallel with the vitamin A tests of the irradiated product litter mates of those animals were fed identically the same amounts of the non-irradiated extracts. The results of feeding the irradiated and non-irradiated products are shown in Table IV.

The vitamin A as well as the color of the carrot extracts had been practically all destroyed by this treatment with ultra-violet light. Rats receiving the non-irradiated extracts made good growth and remained in very good physical condition throughout the entire experimental period while the animals receiving the same amount of irradiated product made no growth, had the appearance of animals receiving the vitamin A-free diet only, and died within a relatively short time.

The behavior of vitamin A after its removal from plant tissue, upon exposure to ultra-violet light, is thus found to be quite like that reported for the vitamin A of cod liver oil and butter fat when the two latter are subjected to somewhat similar treatment.

SUMMARY.

The vitamin A of dry plant tissue appears to be susceptible to destruction during periods of storage. A loss in the vitamin A content of dry spinach amounted to approximately 70 per cent upon storage for a period of 12 to 15 months.

The presence of added rancid fat to the dry plant tissue caused a greater amount of destruction of vitamin A in the plant material during the 12 to 15 months of storage than was found to occur in the spinach that contained no added fat.

The vitamin A of various types of plant tissue was found to be readily soluble in, and extractable by, a vegetable oil.

The result of exposing petroleum ether extracts of carrots to ultra-violet light shows that the vitamin A from this primary source is readily destroyed by such treatment.

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A STUDY OF SOME OF THE CHEMICAL CHARACTER-
ISTICS AND THE ABSORPTION SPECTRUM
OF CYSTINE.

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The present study was undertaken primarily to corroborate
the work of Ward (1), who suggested from data obtained during
the study of the absorption spectra of some biochemical products
that cystine might possess a ring structure rather than an ali-
phatic structure. In the course of the preparation of cystine for
this investigation some interesting results on the relation between
the concentration of the cystine and the pH value at which it
crystallizes or precipitates were obtained.

Cystine was prepared from wool by acid hydrolysis according
to the method of Folin (2) with certain modifications suggested
by Gortner and Sinclair (3). The best yield of crude cystine was
obtained at pH 4 (4). This crude cystine was then dissolved in
the least possible amount of 3 per cent hydrochloric acid, per-
fectly decolorized, and pure cystine in the familiar hexagonal,
plate crystals was obtained. At a pH value of 4 to 5 some of the
same crude cystine gave abundant powdery spherical bodies, which
under the high power microscope resolved themselves into thin,
blunt prisms. These were probably the isomeric form obtained
by Gortner on boiling the crude cystine for a prolonged time in
hydrochloric acid. It is known that cystine can be precipitated
by the use of sodium acetate from solutions of varying hydrogen
ion concentrations (4), a fact which accounts for the differences
noted in the literature on cystine. A precipitation of a white
powder, which resolved itself into very fine needles, was always
obtained at a pH value of 5 to 6.

It was found that there was a direct ratio between the concentration of the cystine and the pH value at which it crystallized, or was precipitated. Different quantities of purified cystine were dissolved in 20 cc. of 3 per cent hydrochloric acid, 0.1, 0.2, 0.3, and 0.4 gm. To each were added 5 cc. of concentrated solution of sodium acetate. Only the most concentrated solution precipitated. Upon addition of 5 cc. to the remaining, unprecipitated solutions, the one containing 0.3 gm. of cystine precipitated.

TABLE I.

pH Values at Which Different Concentrations of Cystine Precipitate.

Sample No.	Cystine.	Concentration of HCl.	pH value.
	gm.	per cent	
I	0.4	3	Strong acid.
II	0.3	3	4
III	0.2	3	5.2
IV	0.1	3	6.5
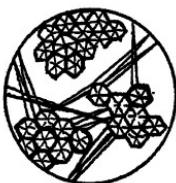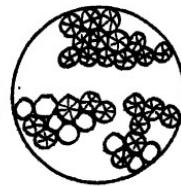
FIG. 1.   FIG. 2.   FIG. 3.

FIG. 1. Character of cystine crystals obtained in strong acid.
FIG. 2. Character of cystine crystals obtained at pH = 3.
FIG. 3. Character of cystine crystals obtained at pH = 5.

Further additions of sodium acetate were needed to bring about precipitation in the other solutions, as shown in Table I.

The character of the crystals obtained from solutions of different pH values is illustrated in Figs. 1 to 3.

Ward's proposal (1) that the structure of cystine is not that usually assumed, that of the aliphatic group, but a ring structure, was based on the fact that the magnitude of the molecular absorption coefficients of cystine in the region of the ultra-violet spectrum studied were nearly as large as those found for such

substances as tyrosine, tryptophane, and phenylalanine, which are known to have ring structure, and much larger than for alanine and glutaminic acid, which have chain structure. On this basis he proposed the following formula for the structure of cystine.

```
        H    H    H    H          .
        |    |    |    |
  H — C — S — S — C — H
        |    |    |    |
  H — C — N — N — C — H
        |    |    |    |
      COOH   H    H  COOH
```
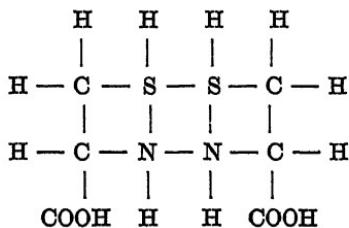
An examination of his curves, however, shows that each of the group of substances which has ring structure, produces selective absorption, a phenomenon characteristic of other substances with that structure, as has been found, for example, by van Gulick (5) for chlorophyllan, by Henri (6) for anthracene, and by Campbell (7) for uric acid. Amino acids without a ring structure do not possess the property of selective absorption but produce only continuous absorption.

The authors have carried out a similar analysis with the two forms of cystine, which were prepared in our laboratory, using the plate form, l-cystine, dissolved in hydrochloric acid and the needle form, i-cystine, dissolved in water. Different concentrations were used, and in the case of the acid solution the ratio of the molecular concentration of cystine and the percentage of hydrochloric acid was kept constant.

We used a rotating sector photometer with a small quartz spectrograph, furnished by the Gaertner Scientific Corporation. The source of light was a hydrogen discharge tube, constructed for us at the United States Bureau of Standards, which gave a continuous spectrum (8) far into the ultra-violet when run by a 1 kilowatt transformer at a pressure of several mm. Because of the homogeneity and intensity of this source it is preferred to the many lined spectrum produced in the under water spark commonly used. A Baly tube with quartz end-plates was filled with the solution and placed between the source and the lower sector of fixed aperture, and a similar tube filled with the solvent was placed between the source and the upper sector of variable aperture.

The molecular absorption coefficient represents the percentage of the light absorbed per cm. of path by a liquid of unit molecular concentration. It was calculated in the usual way from the formula

$$K = \frac{\alpha}{d\,C}$$
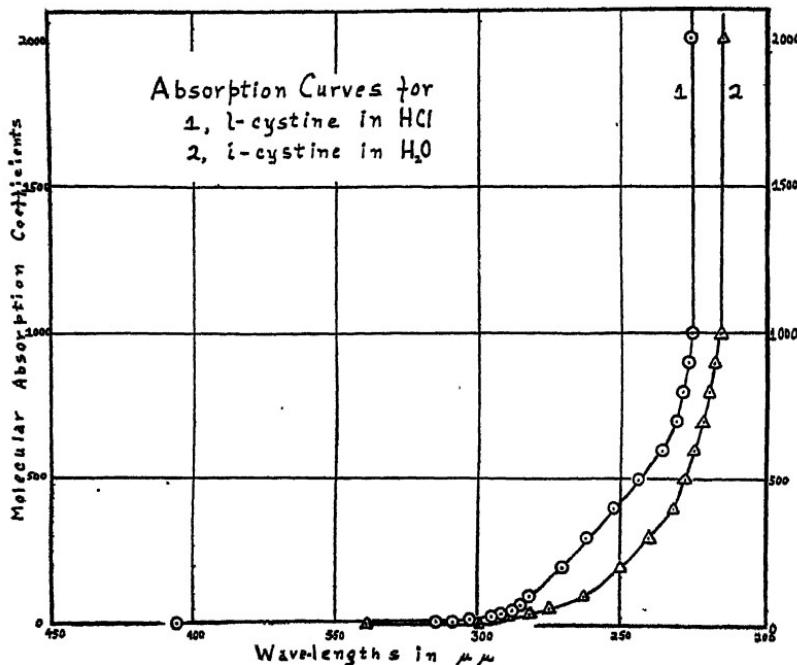
FIG. 4.

where K is the molecular absorption coefficient, α the extinction coefficient read directly on the adjustable sector of the photometer, and C the molecular concentration.

Our results are shown in Fig. 4 where we have plotted the molecular absorption coefficient against the wave-length of the light. The curves obtained when the plate form of cystine was dissolved in different percentages of hydrochloric acid were practically identical, and agree with that obtained by Ward within

experimental error. His values were plotted on a logarithmic scale because of their great range which led to the apparent irregularity near 300 $\mu\mu$. As the diagrams show, the curves are perfectly smooth when the values are plotted on a linear scale. Fig. 5 shows the lower part of our curves in greater detail. The curve obtained with needle cystine in water is also perfectly smooth, but lies further in the ultra-violet.

Absorption Curves for
1, l-cystine in HCl
2, l-cystine in H₂O
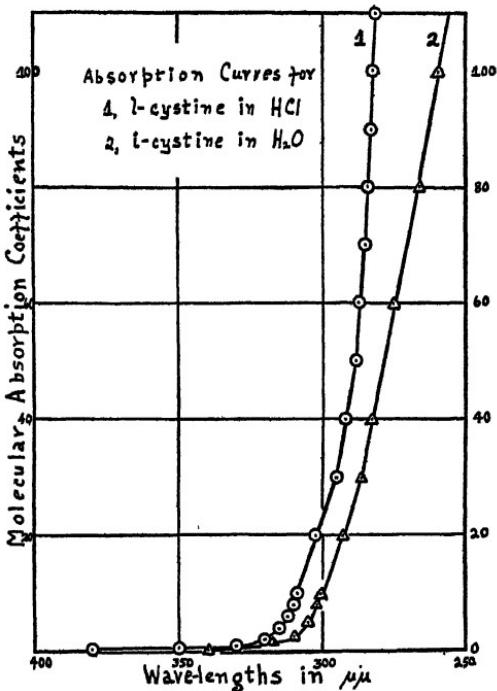
FIG. 5.

We repeated the experiment with alanine, and the results are plotted in Fig. 6. It is of the same type as those obtained with cystine, but begins further in the ultra-violet. Because of the limits of sensitivity of the plates and the length of the path of light it was difficult to carry the measurements further, but the molecular absorption coefficients undoubtedly increase in value as the wave-length of the light absorbed decreases.

The absorption curve of tyrosine, obtained by Ward, has been reproduced in Fig. 7 for purposes of comparison, a linear scale being used in plotting. This shows the character of the selective absorption obtained with substances of ring structure. If cystine has the ring structure suggested by Ward its absorption curve should be similar to this curve, or possibly, like anthracene, should have two regions with selective absorption. If, on the other hand,
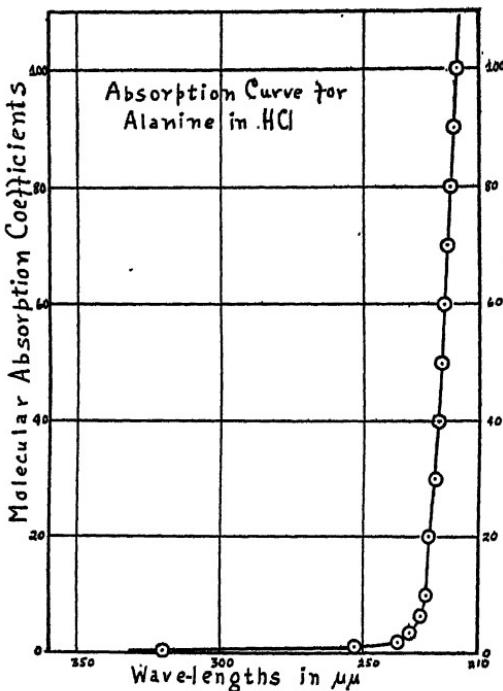
FIG. 6.

it has the chain structure, the absorption curve should be like those of alanine and glutaminic acid (1), which show only continuous absorption. Since the curve is perfectly smooth we conclude that the hypothesis of a ring structure for cystine is untenable.

The continuous absorption shown in the spectrum of cystine is attributed to the dissociation of the molecule. The absorption begins at about 400 $\mu\mu$ in the case of the acid solution and the intensity of the absorption increases as the wave-length of the

light absorbed decreases; *i.e.*, as the energy of the quantum iden-
tified with the light increases. The degree of dissociation may be
calculated by dividing the mass absorption coefficient by the
number of molecules per cm. of path in unit molecular concen-
tration. The value of this coefficient at 400 $\mu\mu$ is approximately
2×10^{-8}, which is of the same order of magnitude as the value
obtained by Sano (9) for the dissociation constant of an acid
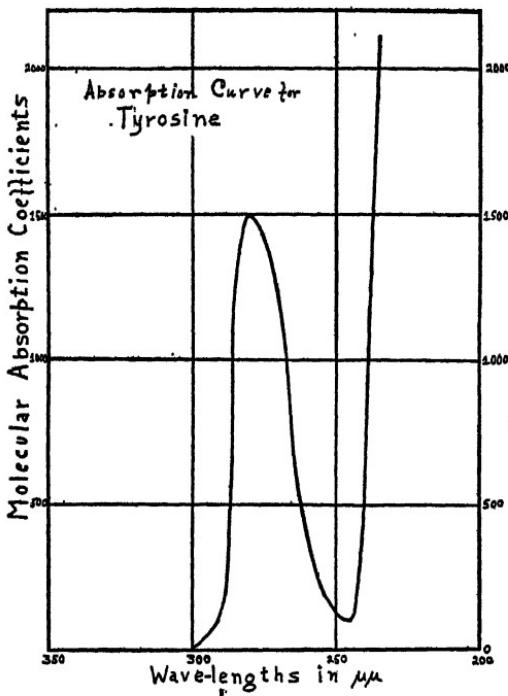
FIG. 7.

solution of cystine under normal conditions. The degree of dis-
sociation produced by light of long wave-length remains small,
10^{-8} to 10^{-7}, probably because there are all possible values for
the angle between the direction of the momentum of the light
quantum and the axis of the molecule, so that in general the
magnitude of the component of the momentum in the direction
necessary to produce dissociation is insufficient. But as the
energy of the quantum increases the probability that this compo-

nent will be sufficient increases, with the result that the degree of dissociation at 226 $\mu\mu$ is over 10^{-4}.

The energy required to produce dissociation may be calculated from the equation, $E = \dfrac{hc}{\lambda}$, where h is Planck's constant, c is the velocity of light, and λ its wave-length in cm. Thus at 400 $\mu\mu$, the wave-length where absorption begins, the energy required to dissociate cystine is calculated to be 4.9×10^{-12} erg per molecule, which corresponds to an ionization potential of 3.7 volts.

The heat of dissociation per mol is accordingly,

$$\frac{E \times \text{No. of molecules per mol}}{J} = 70,800 \text{ calories.}$$

The absorption curve shows that dissociation is complete at 226 $\mu\mu$, which represents an energy of dissociation of 8.7 erg per molecule, or 124,000 calories per mol.

For the cystine dissolved in water, dissociation starts at approximately 340 $\mu\mu$, and is complete at 215 $\mu\mu$, which indicates that slightly more energy is required to dissociate this solution than is required in the case of the acid solution.

Similarly, the energy necessary to produce the dissociation of alanine is larger than that for cystine since the absorption curve lies further in the ultra-violet. This is probably due to the fact that it is less difficult to dissociate cystine since it is a heavier molecule, and since the molecular bond is probably broken between the sulfur atoms, which we know are extremely active.

SUMMARY.

There appears to be a direct ratio between the concentration of the cystine and the pH value at which it crystallizes, or is precipitated.

Since the absorption curves for cystine show only continuous absorption we conclude that the structure of cystine is that usually assumed, a straight chain, and not a ring as proposed by Ward.

From the magnitude of the molecular absorption coefficient we have determined the degree of dissociation, produced by light of different wave-lengths, which ranges from 10^{-8} to 10^{-4}, the former value agreeing with that of Sano.

We have also calculated the dissociation energy which is ap-

proximately 4.9 erg per molecule, or 70,800 calories per gram molecule.

The authors wish to express their appreciation of the assistance and the helpful suggestions given during the spectroscopic examination by Miss Nora Mohler, and to thank Dr. F. L. Mohler of the Bureau of Standards for his kindness in sending us the hydrogen discharge tube used in the investigation.

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ANIMAL CALORIMETRY.

FORTIETH PAPER.

THE METABOLISM OF GLUCOSE ADMINISTERED TO THE FASTING DOG.*

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A number of investigators have reported hyperglycemia and glycosuria ·after carbohydrate ingestion following a fast or a diet deficient in carbohydrates. Hofmeister (1) used the term "hunger diabetes" to describe the glycosuria produced by giving starch to previously fasting animals. Lowered sugar tolerance in rabbits, dogs, and men as a result of fasting for 36 hours or more has been reported by Bang (2), Staub (3), Goldblatt (4), du Vigneaud and Karr (5), and many others, and the similar effect of low carbohydrate diets has been observed by Greenwald, Gross, and Samet (6), Malmros (7), and Tolstoi (8).

The nature of the mechanism for normal utilization of carbohydrates, which is impaired by fasting, has aroused much interest. In view of the value which observations of the respiratory metabolism might have in connection with this problem, it seems remarkable that, in comparison with the large number of sugar tolerance studies, very little attention has been paid to the effect of fasting on the respiratory exchange after sugar ingestion and on the ability of the organism to oxidize carbohydrate. The first observations of the respiratory metabolism in this field were made in 1909 by Johansson (9), who found that when 75 gm. of glucose were given to a man who had fasted from 1½ to 3 days, no increase occurred in the output of CO_2. He attributed this to the retention of the glucose as glycogen. F. G. Benedict's well known Subject L. (10) broke his 31 day fast by taking a diet high in carbohydrates. The metabolism on the 1st day of food was not observed, but the respiratory quotient the next night, beginning 36

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hours after the first meal, was 0.80. The following morning it was 0.78, and on the subsequent night and morning 0.97 and 0.94 respectively.

In 1925 Goldblatt (4) observed that after he had ingested 50 gm. of glucose following a 40 hour fast the R.Q. rose to only 0.81, indicating incomplete carbohydrate oxidation. In apparent contradiction to this Hines, Boyd, and Leese (11), using the continuous injection method of administering glucose to dogs, found that after a fast of 3 to 7 days the hyperglycemia and glycosuria produced by the administration of 4 gm. of glucose per kilo per hour were greater than in the control animals, but the R.Q. approached unity and the heat production was increased 50 per cent.

A seasonal change in rats fasted for 48 hours was found by Cori and Cori (12). In the summer the rate of oxidation of glucose was lower than in winter and there was an increase in excretion of acetone bodies and a slight increase in the blood sugar. Insulin injections brought the oxidation rate up to that observed in winter. Glycogen formation showed no seasonal variation.

Baur (13) studied the effect in men of previous nutrition on the specific dynamic action of 85 gm. of glucose, as measured by an increase in oxygen consumption. He found that the oxygen intake was higher after a carbohydrate-rich diet than after a carbohydrate-poor diet or a fast. However, there was a fairly high specific dynamic action even in a subject whose glycogen stores had been greatly diminished by fasting and exercise. His experiments also included several instances in which there was no proportionality between the specific dynamic action of glucose and the height of the blood sugar, and this led him to question Lusk's theory ((14) pp. 385, 393) that the increase in heat production after carbohydrate ingestion is chiefly caused by a plethora of oxidizable fragments of sugar.

A theory frequently advanced to account for the phenomena of "hunger diabetes" is that there is a gradual decrease in the production of insulin by the pancreas during a period of carbohydrate-want, so that this function is unable to adjust itself at once to the sudden demand occasioned by a plethora of glucose in the blood. Cori and Cori concluded from their experiments and those of Southwood (15) that insulin injections correct this disturbance.

The object of the present investigation is a study of the metabolism of fasting dogs after glucose ingestion, particularly for the purpose of determining the amount of sugar oxidized, a factor not heretofore adequately considered. A comparison of the results obtained with and without accompanying injections of insulin sheds some light on the question of whether or not this hypothesis is adequate to explain the temporary impairment in utilization of glucose.

Methods.

The procedure generally followed involved the fasting of a dog for a period of 19 to 31 days, with exercise on the treadmill in order to deplete the animal's glycogen stores, inasmuch as Anderson and Lusk (16) have shown that after a fast of 13 days a dog may have a non-protein R.Q. of 0.72 while running, indicating that a small amount of glycogen is still stored and available for combustion.

The fasting basal metabolism was determined for a 2 hour period in the respiration calorimeter by the method usually employed in this laboratory. The dog then drank a solution of 50 gm. of glucose in 150 cc. of warm water (37°), and observations of its metabolism were made during the 2nd, 3rd, and 4th hours after glucose ingestion.

Blood was taken from the saphenous vein before the glucose was given and about $4\frac{1}{4}$ hours afterward. Urine was collected by catheterization at about the same time. Overnight urine was also collected in order to follow the urinary nitrogen and to determine quantitatively the total amount of sugar excreted. Figures given for urine sugar in Tables II and IV, with one exception noted later, represent the entire amount of sugar excreted from the time of glucose ingestion until the time when the urine gave a negative reaction to Benedict's test. The urinary nitrogen values given in Tables I and III represent hourly excretion during the periods of the calorimeter observations. Blood sugar determinations were made according to Somogyi's modification of the Shaffer-Hartmann method (17). Urine sugar was determined by Benedict's method and nitrogen by the Kjeldahl method.

Calculations of the amount of sugar oxidized were made as follows: The amount of the heat produced which was due to combustion of carbohydrate was found from the Zuntz-Schumburg-Lusk table ((14) p. 65), calculated from the non-protein R.Q. and oxygen consumed. This was divided by 3.755, the caloric value of 1 gm. of glucose according to Rubner ((14) p. 41). The experimental data consist of a series of observations of basal and glucose metabolism on two dogs, Nos. 71 and 72. A complete day's experiment, as described, was repeated on several successive days with the exceptions noted in the tables. During the entire experimental period the dog received no food except the test meals of glucose.

TABLE I.
Metabolism of Fasting Dog before and after 50 Gm. of Glucose.

Date.	Nature of experiment.	Urine N per hr.	O₂ per hr.	R.Q.	Calories per hr.	
					Indirect.	Direct.

Dog 71. Adult female yellow mongrel.

Date.	Nature of experiment.	Urine N per hr.	O₂ per hr.	R.Q.	Indirect.	Direct.
1930		*gm.*	*gm.*			
Jan. 17*	Basal.	0.083	5.44	0.69	17.75	14.03
	Glucose.	0.130	6.37	0.75	20.88	17.45
" 22	Basal.	0.110	4.90	0.74	16.01	13.53
	Glucose.	0.131	5.52	0.87	18.63	17.11
" 23	Basal.	0.093	4.70	0.75	15.43	12.25
	Glucose.	0.109	5.45	0.94	18.69	15.95
" 30†	Basal.	0.042	4.74	0.89	16.19	11.99
	Glucose.	0.081	6.24	0.94	21.58	17.48
Feb. 6‡	Basal.	0.082	4.55	0.91	15.51	13.39
	Glucose.	0.086	5.88	0.98	20.41	19.34

Dog 72. Adult female mongrel collie.

Date.	Nature of experiment.	Urine N per hr.	O₂ per hr.	R.Q.	Indirect.	Direct.
Feb. 25§	Basal.	0.048	4.85	0.72	15.85	16.16‖
	Glucose.	0.128	5.47	0.74	17.81	17.16
" 26	Basal.	0.025	4.46	0.72	14.65	14.82
	Glucose.	0.053	5.65	0.86	19.18	18.53
" 27	Basal.	0.028	4.36	0.76	14.44	15.62
	Glucose.	0.035	5.28	0.93	18.24	18.30
" 28	Basal.	0.063	4.27	0.78	14.14	14.24
	Glucose.	0.078	5.02	0.95	17.37	16.64
Mar. 6¶	Basal.	0.083	4.31	0.89	14.67	14.55
	Glucose.	0.079	5.33	0.98	18.55	17.38
" 10**	Basal.	0.076	4.23	0.94	14.53	14.53
	Glucose.	0.090	5.69	0.98	19.80	19.30

* 31st day of fast; 6th day after third dose of thyroxine.

† 7th day of maintenance diet.   ‡ 14th day of maintenance diet.

§ 22nd day of fast.

‖ All direct heat values for Dog 72 are without body heat correction.

¶ 6th day of maintenance diet. Basal metabolism calculated on 1 hour only.

** 10th day of maintenance diet.

TABLE II.

Oxidation of Glucose before and after 50 Gm. of Glucose.

Date.	Nature of experiment.	Blood sugar.	Urine sugar.	Cal-ories per hr.	Specific dy-namic action.	Non-protein calories per hr.	Non-protein R.Q.	Glucose oxi-dized per hr.
1930		*mg. per 100 cc.*	*gm.*	*cals.*				*gm.*
Dog 71.								
Jan. 17*	Basal.			17.75†		15.55	0.67†	0
	Glucose.		10.7‡	20.88	3.22	17.43	0.74	0.56
" 22	Basal.	62		16.01		13.09	0.73	0.29
	Glucose.	280	2.2	18.63	2.62	15.16	0.89	2.59
" 23	Basal.	79		15.43		12.96	0.74	0.41
	Glucose.	235	Trace.	18.69	3.26	15.80	0.97	3.80
" 30§	Basal.	72		16.19		15.09	0.90	2.72
	Glucose.	75	0	21.58	5.39	19.43	0.96	4.51
Feb. 6‖	Basal.	117		15.51		13.34	0.93	2.75
	Glucose.	128	0	20.41	4.90	18.13	1.01	4.83
Dog 72.								
Feb. 25¶	Basal.	50		15.85		14.57	0.71	0.04
	Glucose.	350	6.71	17.81	1.96	14.42	0.72	0.18
" 26	Basal.	58		14.65		13.99	0.72	0.18
	Glucose.	252	1.74	19.18	4.53	17.77	0.86	2.56
" 27	Basal.	80		14.44		13.70	0.76	0.70
	Glucose.	146	0	18.24	3.80	17.32	0.94	3.72
" 28	Basal.	79		14.14		12.47	0.77	0.76
	Glucose.	104	0	17.37	3.23	15.30	0.97	3.68
Mar. 6**	Basal.	71		14.67		12.47	0.91	2.35
	Glucose.	81	0	18.55	3.88	16.46	1.00	4.38
" 10††	Basal.			14.53		12.52	0.96	2.96
	Glucose.		0	19.80	5.27	17.43	1.01	4.64

* 31st day of fast.

† If calculated on a diabetic basis, the non-protein R.Q. would be 0.69, the heat 17.66 calories per hour, and the non-protein heat 16.60 calories per hour.

‡ For 4 hours only.   § 7th day of maintenance diet.

‖ 14th day of maintenance diet.   ¶ 22nd day of fast.

** 6th day of maintenance diet. Basal metabolism calculated on 1 hour only.

†† 10th day of maintenance diet.

Control observations were then made on the same animals while they were receiving a ration adequate in respect to carbohydrates and at the same time sufficiently low in protein to keep the basal metabolism at the fasting level. A diet fulfilling these requirements consisted of 50 gm. of meat, 100 gm. of cracker meal, and 20 gm. of lard, daily. The effect of ingested glucose on the metabolism was determined after about a week of this maintenance diet and again about a week later.

Experimental Results.

Data obtained in the respiratory experiments are given in Table I, and the calculations of sugar oxidation and data on hyperglycemia and sugar excretion are presented in Table II.

The most striking fact observed is the almost complete suppression of the oxidation of ingested glucose on the 1st day after a prolonged fast. This is indicated by the fact that the R.Q. rose no higher than 0.75, as compared to 0.98 for the control experiments in which the dogs were receiving a maintenance diet (Table I). The amount of sugar burned per hour was only 4 to 12 per cent of the amount which could be oxidized under normal conditions (Table II).

The hyperglycemia and glycosuria were of the same order of magnitude as those observed by other investigators. Dog 71 (Table II), which was fasted for a longer time than Dog 72, also excreted a larger amount of sugar. The value of 10.7 gm. on January 17 represented only the quantity eliminated during the 4 hours following the glucose ingestion, as a part of the subsequent specimen was lost. Dog 71 had received three intravenous injections of 5 mg. of thyroxine about a week apart, the last injection being 6 days before the first glucose experiment. This animal's weight had been reduced from 15.4 to 9.9 kilos, and that of Dog 72 (Tables I and II) from 13.2 to 10.3 kilos. Glycosuria was lower in Dog 72. 4.2 of the 6.7 gm. were excreted in the urine collected 4 hours after glucose ingestion, and the balance was obtained in the night specimen.

There is a marked similarity in the day to day rate at which the two dogs progressed toward a complete utilization of carbohydrates. The respiratory quotients after glucose ingestion showed a gradual and regular increase. In the case of Dog 72 (Table II)

we obtained the series of non-protein quotients, 0.72, 0.86, 0.94, and 0.97, and with Dog 71 the series 0.74, 0.89, and 0.97, approaching the normal value of 1.00. The amount of glucose oxidized by each dog in the second experiment was about 2.5 gm. This seems remarkable in view of the fact that Dog 72 received glucose on successive days, while a lapse of 5 days occurred between the first and second administration of the sugar to Dog 71. It appears that a 5 day fast did no additional harm to the partially

TABLE III.

Effect of Insulin on Metabolism after 50 Gm. of Glucose.

Dog 71.

Date.	Nature of experiment.	Insulin dose.	Urine N per hr.	O_2 per hr.	R.Q.	Calories.	
						Indirect.	Direct.
1930		*units*	*gm.*	*gm.*			
Apr. 23*	Basal.		0.077	4.59	0.725	14.96	12.41
	Glucose + insulin.†	20	0.093	5.78	0.84	19.43	18.11
" 24	Basal.		0.048	5.21	0.74	17.14	12.15
	Glucose + insulin.	20	0.088	6.09	0.94	21.00	19.87
" 26	" + "	20	0.063	5.67	0.97	19.73	17.25
June 10‡	Basal.		0.096	4.32	0.72	14.12	13.77
	Glucose + insulin.	60	0.125	5.62	0.78	18.68	16.32

* 19th day of fast.

† Average of 2nd and 3rd hours after glucose and insulin.

‡ 22nd day of fast, following 24 days on double standard diet.

restored mechanism for oxidizing carbohydrates and was of insufficient duration to produce the same effect as a 20 day fast.

In the third administration of glucose (Table II) the oxidation rose to about 3.8 gm. per hour, approaching a normal capacity of about 4.5 gm. The hyperglycemia was less marked in each succeeding experiment. The glycosuria was also progressively reduced, so that after the third administration of sugar there was either no sugar or only a trace in the urine.

Both dogs, when given a maintenance diet, showed the ability to oxidize glucose satisfactorily, as indicated by non-protein

respiratory quotients of unity and by the increased heat production (Table II). No glycosuria or hyperglycemia was observed.

The figures in Tables I and II for the specific dynamic action of glucose will be discussed later in this paper.

The effect of insulin was studied in the following experiments. A series of determinations of the basal and glucose metabolism

TABLE IV.

Effect of Insulin on Oxidation of Glucose.

Dog 71. The amounts of insulin and glucose administered are the same as in Table III.

Date.	Nature of experiment.	Blood sugar.	Urine sugar.	Calories per hr.	Specific dynamic action.	Non-protein calories per hr.	Non-protein R.Q.	Glucose oxidized per hr.
1930		*mg. per 100 cc.*	*gm.*	*cals.*				*gm.*
Apr. 23*	Basal.			14.96		12.92	0.71	0
	Glucose + insulin.	220	2.02	19.43	4.47	16.96	0.84	2.13
" 24	Basal.	70	0	17.14		15.87	0.74	0.55
	Glucose + insulin.	116	1.48	21.00	3.86	18.67	0.96	4.34
" 26	" + "	87	0	19.73	4.77†	18.06	0.98	4.50
June 10‡	Basal.			14.12		11.47	0.70	0
	Glucose + insulin.	216	1.77	18.68	4.56	15.23	0.78	1.07

* 19th day of fast.

† Calculated from basal metabolism of April 23.

‡ 22nd day of fast, following 24 days on double standard diet.

was made on Dog 71 after an intervening period of normal nutrition during which the dog regained a weight of 14.6 kilos; this period was followed by a second fast in which its weight was reduced to 11.4 kilos. The experiments were performed exactly as previously described except that, at the time of ingestion of glucose, a subcutaneous injection of 20 units of insulin was given.[1]

[1] The insulin used was U-20 iletin generously furnished by Eli Lilly and Company.

Insulin administered at the time of giving glucose to a fasting dog did not appear to restore completely the animal's normal carbohydrate metabolism. Hyperglycemia was observed on the 1st day of sugar administration and insulin injection, and glycosuria on both the 1st and 2nd days. However, the fact that the insulin aided in some degree the impaired function is indicated by an R.Q. of 0.84 and by the oxidation of 2.13 gm. of glucose per hour. The ability to oxidize sugar appeared to be about the same on the *1st* day of glucose and insulin administration (Table IV) as it had been on the *2nd* day when glucose alone was given after a prolonged fast (Table II). The animal's blood sugar, 220 gm. per 100 cc., corresponds to 280 for the same dog and 252 for Dog 72 after the second glucose ingestion; it excreted 2.02 gm. of glucose,

TABLE V.

Effect of Insulin on Sugar Tolerance of Normal Dogs.

Dog No.	Insulin dose.	Food.	Blood sugar, mg. per 100 cc.			
			Before food.	½ hr. after.	1 hr. after.	2 hrs. after.
	units					
12	None.	100 gm. glucose.	84	126	114	99
12	60	Standard diet.	60		64	64
10	40	" "			78	78

as compared with 2.2 and 1.74, and the amount of sugar oxidized was only slightly less than the 2.59 gm. and 2.56 gm. burned in the other two experiments.

The improvement in carbohydrate utilization on subsequent days took place slightly more rapidly than in the experiments in which no insulin was given. The amount of glucose oxidized was much nearer the normal value on the 2nd day of insulin and glucose than it had been in the third experiment with glucose alone, and the hyperglycemia was much reduced. However, the glycosuria was practically as high as in the corresponding experiment without insulin, and the R.Q. was somewhat lower than that obtained when carbohydrate combustion was normal. It was evident that insulin injections restored a part, but not all, of the usual mechanism for sugar oxidation.

It was thought that possibly 20 units of insulin might have been

insufficient to enable the dog to oxidize 50 gm. of glucose.   Table V[2] shows that 40 and 60 units of insulin, given to normally nourished animals with a test meal containing 100 gm. of carbohydrate, maintained the blood sugar at a constant level.   Therefore another experiment, following the usual procedure, was performed on June 10, when the dose of insulin was increased to 60 units (6 units per kilo).   No greater effect was observed with the larger dose than had been found with 20 units (Tables III and IV).

It is of interest to note that the amount of nitrogen excreted during the 24 hours after the first ingestion of glucose was no lower than during the basal period (Tables I and III).   This confirms the results of Cathcart (18).   Further data on the nitrogen-sparing action of glucose in the fasting dog will be published in a later paper.

Alcohol Checks.

The accuracy of the calorimeter during the period of this investigation was established by eight alcohol checks of 4 hours each (Experiments 288 to 295, inclusive) between January 2 and June 3. The average R.Q. for the eight experiments was 0.662, with a variation between 0.657 and 0.671.   A close agreement between the calculated and the measured heat production was found, the average calories per hour being 25.14 for the indirect method and 25.17 for the direct method of determination.

DISCUSSION.

A fast of 19 to 31 days produces in dogs a temporary but very decided depression of the carbohydrate metabolism.   The suppression of oxidation of glucose, which we observed when this sugar was administered, was accompanied by hyperglycemia and glycosuria.   This was in harmony with the results of other investigators who had studied the effects of shorter fasts on dogs, rats, rabbits, and men.

Fully as striking as the absence of carbohydrate oxidation in the first experiment was the improvement in this function after the fast was broken by the test meal of glucose.   The effect of one dose of 50 gm. of glucose, with no other food or treatment, was

[2] We are indebted to Dr. Margaret A. Kennard for permission to use this table of unpublished data from experiments performed in this laboratory.

such that in the next experiment, either 1 or 5 days later, the oxidative mechanism was somewhat more than one-half as efficient as in the control experiments on normal dogs. Three such doses brought the efficiency to approximately 80 per cent.

In the first experiment following fasting the comparatively small amount of the ingested sugar which could be accounted for by oxidation and excretion is worthy of attention. For example, Dog 72 (Table II) excreted only 6.7 of the first 50 gm. of glucose and oxidized only 0.18 gm. per hour during the 2nd, 3rd, and 4th hours following the ingestion. If oxidation had continued at this rate for 24 hours (a reasonable supposition, in view of the basal experiment of the following morning), the total glucose burned would have been about 4.3 gm., leaving 39 out of the 50 gm. ingested to be accounted for by storage in some form in the tissues.

The experiments of Folin, Trimble, and Newman (19) emphasized the fact that glucose may be temporarily stored as such in the body, with a distribution differing greatly from that usually found for glycogen. They found that the skin of dogs which had received intravenous injections of glucose contained the sugar in as high a concentration as the blood, and the spleen and other organs also contained large amounts. It required about 4 hours for the sugar to be either oxidized, or converted into glycogen and stored in the liver and muscles.

It seems probable that, in the condition of impaired carbohydrate metabolism which we have observed, the time required for this preliminary storage, shift in distribution, and conversion into glycogen, may be greatly prolonged. The fact that the blood sugar, 4 hours after glucose ingestion, was 360 mg., indicates such a delay, just as the return of the blood sugar to 58 mg. the following morning indicates that the process of glycogen storage was probably complete.

The fasting dog's inability to oxidize glucose, the loss of part of the ingested sugar in the urine, and the hyperglycemia, indicating a delay in glycogen synthesis, present a picture which immediately suggests a lack of insulin. One might expect insulin in a fairly large dose to correct these symptoms. As a matter of fact, an injection of 2 or of 6 units per kilo was found to compensate only partially for the effects of fasting. One such dose produced about the same improvement as one dose of 50 gm. of sugar; *i.e.*, roughly

50 per cent efficiency in carbohydrate oxidation. Our results with insulin do not agree entirely with those of du Vigneaud and Karr (5) and of Cori and Cori (12). The former authors reported a decreased tolerance in fasting rabbits to glucose given after recovery from insulin hypoglycemia. The latter found, after one dose of insulin, complete restoration of sugar oxidation in rats fasted for 48 hours. Species differences and the length of the fasting period may account in part for these discrepancies. Southwood (15), in a single insulin experiment on himself after 36 hours of carbohydrate-free diet, observed a sugar tolerance which was inferior to that found while he was partaking of a mixed diet, although much greater than after a 36 hour fast when no insulin was given.

The fact that in our experiments insulin injection did not result in a complete restoration of the power to utilize carbohydrate suggests that prolonged fasting may produce chemical changes in the tissues which are unfavorable to glucose oxidation.

The question arises as to the extent to which acidosis enters into the true explanation. Elias and Kolb (20) attempted to account for "hunger diabetes" on this basis, while Malmros (7) has presented evidence to the contrary. Du Vigneaud and Karr (5) found that administration of sodium bicarbonate counteracted the effect of fasting on the sugar tolerance of rabbits, although the pH and the CO_2-combining power of the blood of these animals was not appreciably lowered by a 4 day fast.

Staub (3), Goldblatt (4), Sevringhaus (21), and others have studied the ketonuria which accompanies hyperglycemia in human subjects when sugar tolerance tests are made after fasting, and Cori and Cori (12) have observed it in rats. It is possible that a condition of acidosis, or the presence of specific products of endogenous metabolism, may interfere with carbohydrate oxidation.

The specific dynamic action of 50 gm. of glucose (Table II) in the dogs on a maintenance diet was found to be about 5 calories hourly, or 32 to 36 per cent of the basal metabolism. This agrees with the value of 30 per cent reported by Lusk ((14) p. 381) for dogs on a standard diet. In the first experiment following the fast the increase in heat production after glucose ingestion was 12 per cent in one dog and 18 per cent in the other, even though very little of the sugar was burned.

The basal metabolism of both dogs while fasting was at least

1 calorie higher than on any day after the fast had been broken. Although the significance of this fact is not known, attention is called to it because these values are used in calculating the specific dynamic action for the first glucose ingested. Calculated on the average basal metabolism of the succeeding days, the first specific dynamic action in Dog 71, instead of 3.22 calories (Table II), would be 5.16 calories, or 33 per cent, and in Dog 72, 3.4 calories, or 24 per cent of the basal heat production.

The heat measured and that calculated on the basis of the oxygen consumption in Dog 71 (Table I) show a discrepancy for which we are unable to account. The average indirect heat in the alcohol checks on January 20 and 21 was 23.51 calories per hour compared to a direct heat of 23.30 calories, indicating that the apparatus was in good condition. The constancy of the difference was such that if the specific dynamic action had been calculated on the direct heat, instead of in the usual manner on the indirect heat, the values would have been quite similar to those given in Table II.

Although there was a slight increase in the specific dynamic action as the glucose oxidation progressively improved, it was not at all proportional to this factor. For example, in Dog 71 (Table II) the specific dynamic action was only slightly lower when no glucose was oxidized than when nearly 4 gm. of glucose per hour were burned.

A comparison between the height of the blood sugar and the increase in heat production in these experiments shows that in the fasting condition there is no relationship between the amount of the specific dynamic action and a plethora of glucose molecules in the blood. For example, in the experiment in which the blood sugar of Dog 72 (Table II) rose to 350 mg. per cent, the specific dynamic action was only 1.96 calories, and in a later experiment when the blood sugar was only 81 mg., the increase in heat production was 3.88 calories.

SUMMARY.

Almost complete suppression of the ability to oxidize ingested glucose, as well as the presence of hyperglycemia and glycosuria, was observed in dogs after a fast of about 3 weeks. In one experiment the non-protein R.Q. before sugar administration was 0.71, and after glucose ingestion only 0.72.

Ingestion of 50 gm. of glucose, even when followed by 5 days of additional fasting, resulted approximately in a 50 per cent restoration of normal carbohydrate utilization. On 4 days of successive administrations of 50 gm. of glucose the non-protein respiratory quotients after glucose were 0.72, 0.86, 0.94, and 0.97, while that of the control animal obtained under normal basal conditions was 1.01.

Insulin injected at the time of glucose ingestion produced only a partial compensation for the loss of the ability to metabolize carbohydrate. This suggests that through disuse the sugar-oxidizing mechanism in the tissues has undergone some change.

Glucose ingested by fasting animals exerted a specific dynamic action which was not dependent upon either the amount of sugar burned or the concentration of glucose in the blood and was probably due to heat production in the intermediary metabolism.

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BILE SALT METABOLISM.

V. CASEIN, EGG ALBUMIN, EGG YOLK, BLOOD, AND MEAT PROTEINS AS DIET FACTORS.

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The experiments given below continue the experimental program of Smith, Groth, and Whipple (1). It will be observed in the tables below that some familiar proteins (casein and beef muscle) given in the diet are favorable for abundant bile salt production but other proteins (egg albumin and gelatin) given in the diet are not favorable for bile salt production. The make-up of these various proteins is fairly well established although some of the data as to amino acids are lacking and some possibly inaccurate. It would seem that one could study the composition tables of these various proteins and decide what amino acids are most essential for bile salt construction within the body. Unfortunately such is not the case, as will be seen in Paper VI of this series, but some information is obtained as to the importance of proline, tryptophane, and glycine, among others.

Methods.

All procedures have been fully described in Paper I of this series (1) and that paper should be consulted for all details. It may be emphasized that meticulous care and constant attention of a skilled technician are essential for satisfactory experimental work with this type of bile fistula.

In brief, bile is collected through a cannula placed in the common bile duct, is brought to the outside through rubber tubes, and is collected in a rubber bag. The bile can be removed from the bag as often as desired and with suitable precautions it is possible to exclude microorganisms. The trunk of the dog is

suitably encased in bandages and a canvas jacket.   With proper care sterile bile can be obtained from the animals for as long as 3 to 4 months.   In our experiments the bile was collected daily, unless otherwise noted, and ordinarily the collection took place between 8 and 10 a.m., usually as close to 9 a.m. as possible.   The figures recorded for any given day pertain to bile which accumulated during the night before and during the afternoon of the preceding day.   Ordinarily the animal was given food during the late morning or early afternoon hours, and as a rule the food was eaten promptly.   Any part which remained uneaten was left in the cage overnight and on the following morning the amount unconsumed was weighed and the amount of food consumed was recorded.

The method for bile salt analysis is a modification of that originally devised by Foster and Hooper (2).   The method is based upon the fact that alkaline hydrolysis breaks down the taurocholic acid and makes available amino nitrogen for estimation by the method of Van Slyke.   Proper correction must be made for the amount of free amino nitrogen present normally in bile.   The amount of correction may be ascertained by a control analysis of unhydrolyzed bile.   .

The salmon-bread used in certain control periods is a complete ration for a normal dog and will maintain such dogs in health indefinitely.   Its preparation and composition are described elsewhere (3).   It contains 1.16 gm. of nitrogen per 100 gm.   To this bread is added 10 per cent by weight of canned salmon and the whole is thoroughly mixed with water to make a mash so that the dogs cannot pick out salmon in preference to bread. When meat products are fed (steak, liver, sweetbreads, etc.) the tissue is cooked in boiling water, ground in a meat chopper, and fed with the broth.   The weight after cooking is given in the tables.   The weight of the broth is not included in the figures.

In all tables it is obvious that *the bile salt output is related to the diet of the day preceding.*   We preferred to record the figures as actually obtained, but in reading the tables, it should be kept in mind that any diet intake is reflected in the bile salt output of the following day.   When a dog has been on a favorable diet (meat) for several days and is placed on an unfavorable diet (sugar, egg white) we note a rapid fall in bile salt output but the

base-line level may not be reached for 2 or 3 days. We assume there may be storage somewhere in the body of substances which are used for bile salt production during the unfavorable diet period, until these reserves are exhausted. Conversely when the dog is changed from an unfavorable to a favorable diet we may note a lag of a day or so before the high plateau level is attained. This may mean that the reserves are repleted before the maximum bile salt output is reached.

Bile cultures were taken every week or 2 or more frequently if there were indications of infection.[1] The earlier experiments gave sufficient data to show that contamination of the bile with dust-borne spore bearers (*e.g. Bacillus subtilis*) introduces no clinical disturbance and causes no fluctuation in the bile salt output. Infection of the bile with pathogenic bacteria in this type of fistula gives a prompt reaction with loss of weight and definite clinical abnormalities.

Experimental Observations.

It may be recalled that *salmon-bread feeding* gives a relatively constant daily output of *bile salts of 100 mg. per kilo*; maximum and minimum figures are 130 and 80 mg. per kilo, respectively. We observe in Tables 51 and 52 that the salmon-bread diet periods average about 120 mg. of bile salt per kilo per 24 hours, being somewhat higher soon after the fistula operation than after an interval of 2 months.

Casein when supplemented by sugar in the diet (Table 51) shows a prompt and sustained rise in bile salt output to a daily average of 186 mg. per kilo. This bile salt output on casein feeding is even a bit higher than the meat feeding level of 175 mg. per kilo (see Table 53). Exactly the same observations are made in Tables 56 and 57, where in different dogs the casein effect on bile salt output is as potent or even slightly more potent than beef muscle feeding.

Casein and sugar made into a paste are fed by hand and are not particularly pleasant to the dogs but there is no loss in weight and the clinical condition is good. The casein used in all our experiments is a commercial casein supplied by Atterbury Broth-

[1] We are indebted to Dr. James A. Kennedy of the Department of Bacteriology for these cultures.

ers, Incorporated, New York City.   There is a moderate chola-
gogue effect in spite of the sugar which tends to cause bile con-
centration (Table 51).   The fall in bile volume at the end of the
feeding period is probably due in part to decreased casein intake.
This is apparent when we compare other experiments with full
diet intake of casein (Tables 56 and 57).

Egg albumin is in direct contrast to casein, and, when given as
the diet protein, shows a very *low bile salt output* (Table 52).   We

TABLE 51.

Casein and Salmon-Bread Feeding.

Dog 28-87.

Date.	Wt.	Diet.			Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
						In 1 cc. bile.	Total output.	
	kg.	*gm.*			*cc.*	*mg.*	*mg.*	*mg.*
1–6		Salmon-bread	440					
7	10.4	"	335		118	0.29	34	1250
8	10.3	"	280		113	0.31	35	1290
9	10.0	Casein 200, sugar	100		122	0.32	39	1430
10	9.9	"	175,	" 125	121	0.39	47	1730
11	9.8	"	175,	" 125	130	0.47	61	2240
12	10.0	"	175,	" 125	105	0.50	52	1910
13	10.3	"	150,	" 105	76	0.68	52	1910
14	9.9				52	0.78	41	1500

Bile fistula operation December 20.
Bile infected; but dog in good clinical condition.
Bile salt average per 24 hours.
  Casein = 186 mg. per kilo.
  Salmon-bread = 130 mg. per kilo.

recall (1) that during fasting periods the bile salt output falls to a
level of 30 to 40 mg. per kilo and we speak of this figure as the
endogenous part of the bile salt output.   We believe this endog-
enous fraction comes at least in part from body protein.

Table 52 illustrates well the low level of bile salt output on a
diet of egg albumin and sugar.   This dog puts out 48 mg. per kilo
per 24 hours on a diet intake of 110 gm. of egg albumin and 150 gm.
of cane-sugar given daily.   The clinical condition of the dog is
excellent and there are minimal weight fluctuations.   Three other

experiments of similar nature are recorded on other dogs in Paper VI, Tables 63, 66, and 67. The bile salt output per 24 hours in these experiments reads 43, 60, and 47 mg. per kilo or an *average of 50 mg. per kilo* on this liberal intake of egg albumin. This albumin intake is ample for maintenance of these fistula dogs and amounts approximately to twenty-five egg whites. Egg albumin (Merck), a fine white soluble powder, was used in all experiments. It was dissolved in water and given with the sugar by stomach tube. Cane-sugar was used in all these experiments. This average for egg albumin of 50 mg. of bile salt per kilo is to be compared with the endogenous part of bile salt secretion, 30 to 40 mg., which leaves us only about 15 mg. of bile salt per kilo attributable to the albumin feeding. We may contrast this very modest output with the generous output due to liver or kidney feeding which might exceed the endogenous fraction by 200 mg. of bile salt per kilo. The contrast with casein is also striking as the casein output may exceed the endogenous fraction by an average of 120 mg. of bile salt per kilo. We may say then that *casein and meat products* may exceed *8 to 15 times* the potency of *egg albumin* as measured in terms of bile salt output per kilo per 24 hours, over and above the endogenous part of the bile salt output as measured in fasting or sugar feeding periods.

Egg yolk feeding is also given in Table 52. This heroic diet intake of egg yolk is maximal and amounted to the cooked yolks of 75 eggs given during 4 days. The egg yolk feeding increased slightly the bile salt output from the salmon-bread level of 120 mg. per kilo to 136 mg. per kilo. All experimental evidence (4) indicates that cholesterol is not concerned in this bile salt reaction. The contrast with egg albumin is striking but we believe this difference will be found to be due to differences in protein constitution.

Egg albumin when added to salmon-bread (Table 52) causes a drop in the bile salt output almost to the low level due to egg albumin and sugar, 57 mg. of bile salt output per kilo. This was a totally unexpected observation, and unfortunately at present unconfirmed. If this is confirmed by other experiments it will indicate that egg albumin contains a substance which inhibits the bile salt output. There is no decrease in total bile flow. So far we have no similar observations other than on certain chemicals

TABLE 52.

Egg Albumin and Egg Yolk Feeding.

Dog 28-87.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
1–30		Salmon-bread 210				
31	9.8	" 220	110	0.27	30	1100
2–1	9.8	" 220	96	0.30	29	1060
2	9.9	" 220	93	0.38	35	1290
3	10.0	S.-bread 220, sugar 150, egg alb. 110	99	0.36	36	1320
4	10.2	" 220, " 150, " " 110	92	0.24	22	810
5	10.3	" 220, " 150, " " 110	94	0.15	14	500
6	10.5	" 220, " 150, " " 110	109	0.15	16	580
7	10.7		98	0.13	13	470
2–9		Salmon-bread 220				
10	10.4	" 220	120	0.30	36	1320
11	10.3	Egg albumin 110, sugar 150	104	0.28	29	1060
12	10.1	" " 110, " 150	37	0.47	17	620
13	10.1	" " 110, " 150	21	0.23	5	184
14	10.1	" " 110, " 150	97	0.14	14	510
15	10.0	" " 110, " 150	72	0.20	14	510
16	10.0	" " 110, " 150	69	0.20	14	510
17	10.0	Salmon-bread 220	70	0.23	16	590
18	10.1	" 220	87	0.28	24	880
19	10.2	" 220	107	0.23	25	920
20	10.3	" 220	125	0.23	29	1070
21	10.5	Egg yolk 180, sugar 150	113	0.27	30	1100
22	10.3	" " 445, " 150	98	0.35	24	1250
23	10.5	" " 265, " 100	126	0.36	45	1650
24	10.1	" " 430, " 150	96	0.40	38	1390
25	9.9		82	0.42	34	1250

Bile fistula operation December 20.
Bile contained bacteria; but dog in good clinical condition.
Bile salt average per 24 hours.
  Egg albumin + sugar = 48 mg. per kilo.
  Egg albumin + salmon-bread = 57 mg. per kilo.
  Egg yolk + sugar = 136 mg. per kilo.
  Salmon-bread = 110 mg. per kilo.

(indene) which may inhibit bile salt output, but the dog is clinically disturbed and probably slightly poisoned. This question of inhibition calls for much more study.

TABLE 53.

Sweetbreads and Beef Muscle Feeding.

Subject.	Date.	Wt.	Diet.		Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
						In 1 cc. bile.	Total output.	
		kg.	*gm.*		*cc.*	*mg.*	*mg.*	*mg.*
Dog 28-87.	3–18		Sweetbreads 350					
	19	10.8	"	350	102	0.29	30	1100
	20	10.5	"	350	122	0.35	43	1580
	21	10.8	"	350	134	0.37	49	1800
	22	10.5	Beefsteak 350		119	0.43	51	1870
	23	10.5	"	350	101	0.42	42	1540
	24	10.7	"	350	99	0.56	56	2060
	25	10.5	"	350	99	0.46	46	1690
	26	10.4			109	0.51	56	2060
Dog 28-124.	3–11		Salmon-bread 165					
	12	13.7	"	165	111	0.31	35	1280
	13	14.0	"	280	108	0.30	32	1170
	14	14.0	"	350	114	0.33	38	1390
	15	14.2	Sweetbreads 360		95	0.38	36	1320
	16	14.0	"	120	102	0.36	37	1360
	17	13.5	"	290	83	0.43	36	1320
	18	13.1	"	270	60	0.51	31	1140
	19	13.1			69	0.45	31	1140

Bile fistula operation December 20, Dog 28-87.
Bile fistula operation March 7, Dog 28-124.
Bile not sterile but both dogs in good clinical condition.
Bile salt average per 24 hours.
  Sweetbreads = 150 and 92 mg. per kilo.
  Salmon-bread = 92 mg. per kilo.
  Beef muscle = 175 mg. per kilo.

Egg albumin feeding causes no cholagogue effect in any of these experiments, in fact there is usually ·a fall in total bile output which may be in part due to the sugar intake.

· *Sweetbreads* are tested in Table 53 and compared with salmon-bread and beef muscle. We may refer to a third experiment given in Table 57. *Sweetbreads* as purchased in the open market

offer a surprizing variety, and besides pancreas include a liberal percentage of thymus and lymph glands, as established by histological sections.   The sweetbreads were cooked in boiling water, and the broth was fed with the cooked glands.   The weight *after* cooking is given in Tables 53 and 57.   The weight of the broth is not included in the figures given.   We felt that this mixed material was satisfactory as we wished to test gland nucleic acids for their influence on bile salt output.   We have tested previously (4) the effect of yeast nucleic acids and have found them to be inert. Gland nucleic acids also present nothing unusual and sweetbread feeding does not measure up to the potency of beef muscle.

Sweetbreads fed in liberal amounts to bile fistula dogs present considerable variety of output of bile salts.   The first experiment (Table 53, Dog 28-87) shows a bile salt output per 24 hours of 150 mg. per kilo, which is lower than the beef muscle level of 175 mg. per kilo.   The next experiment (Table 53, Dog 28-124) shows a lower output of bile salts of 92 mg. per kilo which is identical with the bile salt output on standard salmon-bread. The third experiment with sweetbreads (Table 57, Dog 29-8) with maximal intake shows an even lower bile salt output of 77 mg. per kilo which is below the level for salmon-bread feeding, or 90 mg. of bile salt per kilo.   This last dog shows a low level for all meat products in spite of good clinical condition, liberal food intake, constant body weight, and sterile bile.   We observe such dogs not infrequently and this fact makes adequate standardization of each new bile fistula dog an obvious necessity.

Sweetbreads show no unusual cholagogue effect and are to be placed between salmon-bread and beef muscle in this respect. The variation in potency may be explained by the motley assortment of gland substances.   There is no reason to suspect that *gland nucleic acids* are unusual when given to bile fistula dogs as measured by bile flow or bile acid output.

Red blood cells (Table 54) fed in large amounts to bile fistula dogs do not cause any unusual output of bile salts.   The blood cells are prepared as follows: Beef blood is whipped free of fibrin, which removes most of the white cells.   The blood is then centrifugalized and all the serum poured off.   The red cells, 4000 cc., are then put in a double boiler and cooked thoroughly.   This yields 3920 gm. of crumbly dark brown material which is fed in

amounts as indicated in Tables 54 to 56. The dog shows some loss of weight during this diet period, probably wholly due to inadequate food supply, as a considerable part of the hemoglobin is passed through the intestinal tract incompletely digested.

Table 54 shows that red blood cells in liberal amounts in the diet yield an output of bile salts of 83 mg. per kilo or slightly less than the output level on salmon-bread of 99 mg. per kilo. Table 56 likewise shows an identical experiment in which the blood cell output level is 110 mg. per kilo but the salmon-bread level is

TABLE 54.

Red Blood Cells Fed.

Dog 29-8.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
4–28		Salmon-bread 440				
29	15.9	" 440	60	0.71	42	1550
30	16.0	Cooked blood cells 490	68	0.68	46	1690
5–1	15.4	" " " 210	96	0.55	53	1940
2	15.0	" " " 490	35	0.74	26	950
3	15.0	" " " 490	44	0.75	33	1210
4	14.9	Salmon-bread 440	50	0.79	39	1430
5	15.0	" 440	33	1.06	35	1280
6	15.7		66	0.70	46	1690

Bile fistula operation February 20. Bile sterile.
Bile salt average per 24 hours.
  Red blood cells = 83 mg. per kilo.
  Salmon-bread = 99 mg. per kilo.

116 mg. per kilo. There is nothing unusual about the total output of whole bile which does not exceed the usual salmon-bread level.

Whole blood (Table 55) supplements salmon-bread to give high output figures for bile salts. This contrasts with the negative effect of red blood cells alone (Table 54). Two experiments with whole blood (Table 55) were performed on the same dog which showed a higher bile salt output in the second experiment. The salt output was 116 mg. per kilo in the first experiment and 144 mg. per kilo in the second, while the control salmon-bread output was

86 mg. per kilo.   It is obvious that whole beef blood supplements the salmon-bread but we cannot say what effect the whole blood alone might have.   The blood serum proteins obviously may be concerned and this point must be established.   Whole beef blood was cooked in a double boiler and the coagulum mixed with the

TABLE 55.

Whole Beef Blood and Salmon-Bread Feeding.

Dog 28-124.

Date.	Wt.	Diet.				Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
							In 1 cc. bile.	Total output.	
	kg.	*gm.*				*cc.*	*mg.*	*mg.*	*mg.*
4–8		Salmon-bread 125, cooked blood 150							
9	12.6	"	225,	"	"	150	102	0.36	37	1360
10	12.5	"	200,	"	"	175	108	0.38	41	1500
11	12.5	"	200,	"	"	175	93	0.44	41	1500
12	12.6	"	225				92	0.42	39	1430
13	12.4	"	250				62	0.51	32	1170
14	12.4	"	250				55	0.50	27	990
15	12.3	"	250				68	0.43	29	1070
16	12.4	"	220,	cooked blood 200			75	0.38	28	1030
17	12.5	"	175,	"	"	160	114	0.37	42	1540
18	12.3	"	220,	"	"	200	85	0.53	45	1660
19	12.2	"	220,	"	"	200	125	0.39	49	1800
20	12.6	"	220,	"	"	100	156	0.37	58	2130
21	12.7						160	0.36	57	2090

Bile fistula operation March 7.   Bile infected after March 16.
Bile salt average per 24 hours.
  Blood + salmon-bread = 116 mg. per kilo.
  Salmon-bread = 86 mg. per kilo.
  Blood + salmon-bread = 144 mg. per kilo.

bread; the cooked weight is given.   Whole blood plus salmon-bread has a moderate cholagogue effect comparable to beef muscle.

Table 56 presents an uninterrupted series of observations on the same dog, in which we may compare the salmon-bread control periods with casein, steak, and red blood cells.   This dog shows a rather high bile salt output on standard salmon-bread, an average of 116 mg. of bile salt per kilo per 24 hours.   The output on beef

muscle feeding is below average and amounts to 147 mg. per kilo, which is practically the same as in casein feeding which yields

TABLE 56.

Red Blood Cell, Steak, and Casein Diet Periods.

Dog 29-169.

Date.	Wt.	Diet.		Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
					In 1 cc. bile.	Total output.	
	kg.	gm.		cc.	mg.	mg.	mg.
4–10		Salmon-bread 335					
11	12.2	" 195		84	0.46	39	1430
12	12.1	" 255		94	0.42	40	1470
13	12.0	" 230		88	0.43	38	1390
14	11.9	Casein 200, sugar 100		84	0.43	36	1320
15	11.7	" 200, " 100		124	0.37	46	1690
16	11.7	" 200, " 100		104	0.52	54	1980
17	11.9	" 200, " 100		50	0.63	32	1170
18	11.9	Salmon-bread 230		106	0.49	52	1910
19	11.7	" 360		80	0.51	41	1500
20	12.3	" 420		86	0.43	37	1360
21	12.3	Steak 380		88	0.44	39	1430
22	11.9	" 380		136	0.34	46	1690
23	11.8	" 380		124	0.41	51	1870
24	11.7	" 380		106	0.44	47	1720
25	11.7	Salmon-bread 440		91	0.51	46	1690
26	12.1	" 335		68	0.64	44	1610
27	12.2	" 335		80	0.42	33	1210
28	12.2	" 230		62	0.42	26	950
29	12.1	" 260		94	0.46	43	1580
30	12.2	Cooked blood cells 460		90	0.45	41	1500
5–1	12.1	" " " 230		119	0.39	47	1730
2	11.9	" " " 490		68	0.58	39	1430
3	11.6	" " " 490		58	0.46	27	990
4	11.2			32	0.84	27	990

Bile fistula operation February 25. Bile sterile.

Bile salt average per 24 hours.

Salmon-bread = 116 mg. per kilo.

Casein + sugar = 143 mg. per kilo.

Steak = 147 mg. per kilo.

Red blood cells = 110 mg. per kilo.

143 mg. per kilo. The output on beef red blood cells is 110 mg. per kilo. During this period the weight was uniform except for a

TABLE 57.

Digested and Whole Beef Muscle, Sweetbreads, and Casein Feeding.

Dog 29-8.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
-30		Salmon-bread 440				
31	16.1	" 440	58	0.58	34	1250
4-1	15.7	Digested meat (517 gm. equiv.)	50	0.69	34	1250
2	15.6	" " (517 " ")	37	0.86	32	1170
3	15.3	" " (517 " ")	38	0.92	35	1280
4	15.2	" " (517 " ")	31	0.95	29	1060
5	15.2	Salmon-bread 440	36	0.95	34	1250
6	15.7	" 440	38	0.96	36	1320
7	15.9	" 440	52	0.81	42	1540
8	15.8	Sweetbreads 400	62	0.61	38	1390
9	15.3	" 400	66	0.62	41	1500
10	15.2	" 400	48	0.69	33	1210
11	15.0	" 400	34	0.81	28	1030
12	15.0	Salmon-bread 440	31	0.83	26	950
13	15.6	" 440	21	0.98	21	770
14	15.5	Casein 200, sugar 100	42	0.63	26	950
15	15.0	" 200, " 100	83	0.57	47	1720
16	15.0	" 200, " 100	70	0.74	52	1910
17	15.0	" 200, " 100	82	0.71	58	2130
18	14.9	Salmon-bread 440	53	0.88	47	1720
19	15.2	" 440	50	0.93	46	1690
20	15.4	" 440	70	0.78	55	2020
21	15.4	Steak 380	70	0.68	48	1760
22	15.1	" 380	79	0.56	44	1610
23	15.0	" 380	90	0.67	60	2200
24	15.0	" 380	70	0.66	46	1690
25	14.8	Salmon-bread 440	62	0.67	42	1540
26	15.4		53	0.70	37	1360

Bile fistula operation February 20.   Bile sterile.

Bile salt average per 24 hours.

  Salmon-bread = 89 mg. per kilo.

  Digested meat = 78 mg. per kilo.

  Whole meat = 117 mg. per kilo.

  Casein = 125 mg. per kilo.

  Sweetbreads = 77 mg. per kilo.

loss on the red blood cell diet. The clinical condition was excellent and the bile sterile.

Table 57, like Table 56, presents an uninterrupted series of observations on a dog in excellent condition with minimal weight fluctuations and sterile bile. The average level for the salmon-bread diet is 89 mg. of bile salt per kilo per 24 hours. The output on sweetbreads is 77 mg. per kilo, for beef muscle is 117 mg. per kilo, and for casein 125 mg. per kilo. These values have been

TABLE 58.

Digested Meat Broth and Salmon-Bread Feeding.

Dog 29-169.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
3-28		Salmon-bread 220				
29	12.2	" 200	88	0.37	33	1210
30	12.1	" 200	98	0.36	35	1290
31	12.0	" 270	76	0.43	33	1210
4-1	12.0	Digested meat (517 gm. equiv.)	71	0.37	26	950
2	12.0	" " (517 " ")	74	0.42	31	1140
3	11.8	" " (517 " ")	94	0.37	35	1290
4	11.6	" " (517 " ")	64	0.48	31	1140
5	11.8	Salmon-bread 385	70	0.43	30	1100
6	12.1		70	0.54	38	1400

Bile fistula operation February 25. Bile sterile throughout.
Bile salt average per 24 hours.
  Digested meat = 99 mg. per kilo.
  Salmon-bread = 100 mg. per kilo.

discussed above in connection with other tables and similar experiments.

Digested steak also appears in Table 57 and shows an output of 78 mg. of bile salt per kilo. This experiment is discussed below (Table 58).

A steak digest is tested in Table 58 and presents points of interest relating especially to methods of investigation in this field. It will be recalled that beef muscle in the diet favors a high output of bile salt which may be double the output on standard salmon-

bread. With this fact in mind we thought a meat digest might be prepared and fractionated to ascertain the relative potency of the different fractions. We were not prepared for the experimental results which indicate a destruction of much potent material during an artificial pancreatic digestion followed by concentration. We have no satisfactory explanation to offer but will say merely that artificial digestion and concentration appear to destroy some substance so that the digest is scarcely as potent as salmon-bread, a level much below that of whole beef muscle fed as such.

The first experiment with the meat digest was performed with a combined bile and Eck fistula (Table 95, Paper IX). There was a very low bile salt output but we explained this as due to the Eck fistula which renders a dog subject to intoxication during meat diet periods. However it was suggested that tryptophane might be destroyed during concentration of the meat digest due to HCl present, used to neutralize the sodium carbonate added prior to the digestion. Therefore particular care was used in the preparation of the digest used in Tables 58 and 57 to maintain neutrality during concentration of the digest.

The meat digest was prepared as follows: Grind 4540 gm. of beefsteak, free of fat, in a meat chopper. Add 7500 cc. of distilled water and heat to 80°. Add 60 gm. of sodium carbonate and cool to 40°. Add 150 cc. of pancreatic extract, prepared according to the method of Cole and Onslow (5). Add 300 cc. of chloroform. Digest overnight at room temperature. Filter, and add hydrochloric acid to filtrate to neutral reaction. This filtrate was later boiled over a free flame down to smaller volume after sodium hydroxide had been added to restore neutrality to litmus. The residue not digested down weighed 2070 gm., and to this 30 gm. of sodium carbonate, 80 cc. of pancreatic extract, 100 cc. of chloroform, and 4000 cc. of distilled water were added and the mixture digested overnight again. It was filtered and drained as before. The residue now was 1250 gm. This was again digested overnight, this time with 15 gm. of sodium carbonate, 80 cc. of pancreatic extract, 100 cc. of chloroform, 2000 cc. of water. Following this third digestion, 400 gm. of digest residue remained. This was discarded. The combined filtrates, after evaporation, totalled 4936 cc. They were preserved in the ice box and to each dog 617 cc. were fed daily.

The *meat digest* tested in Table 58 shows no more potency for bile salt formation than salmon-bread; in fact the figures are identical—99 mg. per kilo for the meat digest and 100 mg. per kilo for salmon-bread. Moreover the intake was very liberal and amounted to a digest from 517 gm. (if we exclude the part discarded) of beef muscle given each day. There was but slight loss of weight. We note similar results in Table 57 where the bile salt output per day is 89 mg. per kilo on salmon-bread and only 78 mg. per kilo on the meat digest.

The lack of cholagogue effect is significant and probably indicates that amino acids have been destroyed by this method of preparation, as Paper VI shows that certain amino acids have a definite cholagogue effect (see Tables 64 and 65).

DISCUSSION.

In this laboratory during a study of experimental anemia in dogs it has been observed that certain proteins in the diet are very favorable for new hemoglobin production (*e.g.* liver and kidney) while on the contrary other proteins in the diet are relatively inert (*e.g.* casein). One of us (Whipple) long has had the conviction that the liver plays an essential rôle in *constructive* hemoglobin metabolism; in other words the liver effects the initial grouping of amino acids to form *parent hemoglobin materials* which are utilized in the bone marrow in the maturing red blood cells. Positive evidence for this hypothesis will soon be published from this laboratory. With this in mind we studied with interest the effect of proteins favorable for hemoglobin production when given to bile fistula dogs. Liver and kidney feeding give maximal returns of bile acids and this might suggest some parallelism between hemoglobin production and bile salt output. But the casein experiments dispelled this illusion, for this protein is unfavorable for hemoglobin regeneration but, as seen above, is almost on a par with liver, kidney, and beef muscle when measured by its effect on bile salt output. Further experimental evidence on this point will be found in Paper VIII, Table 84.

SUMMARY.

In the bile fistula dog one may measure the potency of any given protein or food substance in terms of bile salt output.

Liver, kidney, and beef muscle fed to bile fistula dogs show a maximal potency and may produce a bile salt output as high as 200 to 300 mg. of bile salt per kilo of body weight per 24 hours.

Casein under identical conditions may show a bile salt output equivalent to low levels for beef muscle feeding. The figures in the published experiments run from a minimum of 125 mg. to a maximum of 190 mg. per kilo of body weight.

Egg albumin in contrast to casein when fed with sugar gives very low output values for bile salts in fistula dogs. The output in four experiments averages 50 mg. of bile salt per kilo per 24 hours.

It will be recalled that the endogenous part of bile salt output as established by fasting and sugar feeding experiments amounts to 30 to 40 mg. of bile salt per kilo. This shows the minimal exogenous effect of egg albumin feeding as measured by bile salt output.

Standard salmon-bread used in control diet periods shows a bile salt output which averages very close to 100 mg. per kilo. Individual dogs show departures from this mean.

Sweetbread feeding gives no evidence that gland nucleic acids are concerned with bile salt metabolism. Sweetbread diet output of bile salt shows average values close to the salmon-bread level and decidedly below casein or beef.

Red blood cells (beef) cooked and fed alone show a bile salt output level slightly below the control level of salmon-bread.

Whole beef blood given with salmon-bread may increase considerably the output of bile salts above the salmon-bread average level. This may indicate that blood plasma proteins are concerned.

A digest of beef muscle shows a loss of potency as compared to whole beef. This may indicate destruction of certain amino acids.

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BILE SALT METABOLISM.

VI. PROLINE, TRYPTOPHANE, AND GLYCINE IN DIET.

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The essential rôle played by proteins in bile salt metabolism has been emphasized in this series of papers, particularly Paper II (1). A study of various amino acids was the next logical step even though it is expensive and time-consuming. The experiments given below indicate that *proline*, like *tryptophane*, may under certain conditions increase the output of bile salts. There is a conspicuous cholagogue effect for which we may choose to place responsibility on the pyrrole ring.

We have submitted evidence (2) to show that tryptophane may be a determining factor in bile salt metabolism. It is recalled that gelatin feeding in a bile fistula dog gives a bile salt output per 24 hours of 70 mg. per kilo. Salmon-bread feeding gives bile salt figures of about 100 mg per kilo. When gelatin is supplemented with tyrosine, no change is observed, but when supplemented with tryptophane, we note a great increase in bile salt production to a level of 180 mg. per kilo. Tryptophane alone causes no increase in bile salts but a conspicuous cholagogue effect. From this evidence we concluded that *tryptophane* combined with other substances could complete the internal cycle of bile salt production.

We refer again to Paper III (2) for the structural formula of cholic acid which is the limiting factor in bile salt production in the body (3). Cholic acid contains two indene rings (combined 6-carbon and 5-carbon rings). Tryptophane contains an indole ring which *physiologically* seems to be related to the indene ring of cholic acid although a replacement of this sort is viewed with alarm by the organic chemist. We do not present this claim as established but await an explanation of the observed

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facts which may be satisfactory to chemist and physiologist alike.

Proline in three respects resembles tryptophane: It contains the pyrrole ring, it causes an increase in bile salt production under certain conditions, and it has a positive cholagogue effect. May we say that the pyrrole ring of proline is concerned with these two physiological reactions?

TABLE 61.

Proline and Standard Salmon-Bread Feeding.

Dog 28-87.

Date.	Wt.	Diet.			Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
						In 1 cc. bile.	Total output.	
	kg.	gm.			cc.	mg.	mg.	mg.
1–20		Salmon-bread	250					
21	9.6	"	220		75	0.44	33	1210
22	9.8	"	220		95	0.30	28	1030
23	9.7	"	220, proline	10	83	0.34	28	1030
24	9.9	"	220, "	10	108	0.34	37	1360
25	9.8	"	220, "	10	112	0.35	39	1430
26	10.2	"	220, "	10	102	0.38	39	1430
27	9.7				108	0.33	36	1320

Bile fistula operation December 20.
Bile infected but dog in good clinical condition.
Bile salt average per 24 hours.
  Salmon-bread = 112 mg. per kilo.
  Proline + salmon-bread = 140 mg. per kilo.

Experimental Observations.

These experimental observations are a continuation of those described in the preceding paper, Paper V, and all conditions are as indicated above. Table 61 shows the effect of adding proline to the salmon-bread control ration. There is a definite increase in bile salt output in the proline feeding period and we note a rise from the salmon-bread level of 112 mg. of bile salt per kilo to 140 mg. of bile salt per kilo during the proline period. There is a distinct cholagogue effect due to the proline feeding.

The proline used in these experiments was prepared in the Laboratory of Synthetic Chemistry of the Eastman Kodak

Company.[1] The proline was prepared from gelatin and contained some unknown impurities and was not in crystalline form. It obviously contained no tryptophane but may have contained traces of other amino acids contained in gelatin. The amounts of proline recorded in Tables 61 to 64 are only approximate and may be a little too high. The proline was given by stomach tube.

Table 62 shows other experiments with proline which are in harmony with Table 61. Proline added to gelatin is nothing like as potent as tryptophane, but it does raise the bile salt output above the low gelatin level. This experiment must be repeated with pure proline because impurities may be responsible for this moderate reaction. Gelatin, of course, contains much proline.

Proline (Table 62) added to salmon-bread causes a strong increase above the salmon-bread base-line, a rise from the salmon-bread level of 101 mg. of bile salt per kilo of body weight to 179 mg. of bile salt per kilo, which high level is a little above the beef muscle output in the same table or 155 mg. of bile salt per kilo. (This output on beef muscle feeding is below the average probably due to a low meat intake.) The apricot feeding is discussed in Paper VIII. Commercial dried apricots were used and the dry weights are given in Table 62.

Cholagogue effect (Table 62) is distinct, although the uptake after proline feeding is somewhat delayed and does not reach a maximum for 2 to 3 days.

Table 63 shows an egg albumin experiment which is discussed in the preceding paper. The proline experiment is not satisfactory because the stomach tube feeding of the proline upset the dog's appetite and we observe a low intake of salmon-bread. The bile salt output in this experiment is not increased by proline feeding but is actually below the control salmon-bread level. There is a moderate cholagogue effect noted at the end of the proline period.

Proline and tryptophane (Table 64) both appear to supplement favorably the egg albumin diet and increase the bile salt output from the egg albumin level of about 50 mg. per kilo to 116 mg. per kilo with proline and 105 mg. per kilo with trypto-

phane.　The salmon-bread level for this dog was unusually high at this time or 152 mg. of bile salt per kilo.　This dog shows an

TABLE 62.

Proline, Gelatin, and Salmon-Bread Feeding.

Dog 28-124.

Date.	Wt.	Diet.			Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
						In 1 cc. bile.	Total output.	
	kg.	*gm.*			*cc.*	*mg.*	*mg.*	*mg.*
3–23		Beefsteak	95					
24	12.9	"	325		134	0.29	39	1430
25	12.7	"	95		172	0.32	55	2020
26	12.5	Salmon-bread 385			155	0.43	67	2460
27	13.1	"	310		124	0.41	51	1870
28	13.3	"	305		93	˙0.39	36	1320
29	13.1	"	190		85	0.35	30	1100
30	12.9	"	440		82	0.36	29	1060
31	13.3	"	285		89	0.43	38	1390
4–1	13.1	Gelatin 225, lard 50			84	0.40	34	1240
2	12.6	" 225, "	50, proline 5		48	0.57	27	990
3	12.2	" 225, "	50, " 5		17	1.22	21	770
4	12.1	" 225, "	50, " 5		21	1.08	23	840
5	12.1				51	0.82	42	1540
4–24		Salmon-bread 230, apricots 200						
25	12.4	"	275, "	200	97	0.37	36	1320
26	12.4	"	275, "	200	94	0.38	36	1320
27	12.4	"	275, "	200	112	0.36	40	1470
28	12.6	"	275, "	200	110	0.35	39	1430
29	12.5	"	330		120	0.31	37	1360
30	12.2	"	330, proline 10		112			
5–1	12.4	"	330, " 10		130	0.30	39	1430
2	12.2	"	330, " 10		140	0.31	44	1620
3					286	0.34	97	3560

Bile fistula operation March 7.　Bile infected after March 16.
Bile salt average per 24 hours.
　Salmon-bread = 101 mg. per kilo.
　Apricots + salmon-bread = 110 mg. per kilo.
　Beefsteak = 155 mg. per kilo.
　Proline + salmon-bread = 179 mg. per kilo.
　Proline + gelatin = 87 mg. per kilo.

unusually large bread intake, was in perfect physical condition, and the bile was sterile.

The cholagogue effect (Table 64) of tryptophane is well shown and is to be compared with usual 24 hour whole bile output of 50 to 60 cc. during egg albumin diet periods. Proline shows less

TABLE 63.

Proline and Egg Albumin in Diet.

Dog 28-46.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
1–20		Salmon-bread 250				
21	10.3	" 250	74	0.47	35	1280
22	10.5	" 125	100	0.36	36	1320
23	10.4	Egg albumin 100, sugar 150	74	0.48	36	1320
24	10.4	" " 100, " 150	63	0.26	16	585
25	10.3	" " 130, " 150	50	0.27	13	475
26		" " 130, " 150	50	0.19	9	330
27	10.5	Salmon-bread 220	41	0.25	10	367
28	10.3	" 330	74			
29	10.4	" 330	138			
30	10.5	" 0, proline 10	129			
31	10.7	" 200, " 10	53	0.42	22	805
2–1	10.2	" 110, " 10	41	0.28	11	405
2	10.3	" 160, " 10	95	0.29	28	1030
3	10.3	" 190, " 10	118	0.31	37	1360
4	10.7	" 70, " 10	132	0.30	40	1470
5	10.3		84	0.37	31	1140

Bile fistula operation December 11. Bile contained bacteria after December 19.

Dog in good clinical condition.

Bile salt average per 24 hours.

Salmon-bread = 127 mg. per kilo.

Proline + salmon-bread = 99 mg. per·kilo.

Egg albumin + sugar = 43 mg. per kilo.

cholagogue effect but the bile flow is almost double that to be expected with egg albumin and sugar alone.

Table 65 shows an experiment with isatin which is discussed in the next paper (Paper VII). The combination of tryptophane, proline, and sugar shows exactly the same reaction as tryptophane and sugar as given in Paper III. There is a fall to the low bile

salt level due to sugar feeding alone; in this dog (Table 65) to 39 mg. of bile salts per kilo. Evidently neither tryptophane nor proline alone or combined can form bile salts but must be supplemented by some other substance or substances.

TABLE 64.

Proline, Tryptophane, and Egg Albumin in Diet.

Dog 28-177.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
6–4		Salmon-bread 440				
5	14.7	" 440	162	0.39	63	2310
6	14.7	" 440	180	0.36	65	2390
7	14.6	Egg alb. 110, sugar 150, proline 4.7	150	0.41	61	2240
8	14.3	" " 110, " 150, " 4.7	108	0.49	53	1940
9	14.4	" " 110, " 150, " 4.7	114	0.41	47	1720
10	14.4	" " 110, " 150, " 4.7	74	0.53	39	1430
11	14.5	" " 110, " 150	79	0.54	43	1580
12	14.4	" " 110, " 150, trypt. 5	54	0.48	26	950
13	14.3	" " 110, " 150, " 5	178	0.30	53	1940
14	14.0	" " 110, " 150, " 5	199	0.15	30	1100
15	14.0	" " 110, " 150	200	0.19	38	1390
16	14.0	Salmon-bread 440	122	0.31	38	1390
17	14.2	" 440	147	0.36	53	1940
18	14.4		169	0.34	58	2130

Bile fistula operation May 14.   Bile sterile throughout.
Bile salt average per 24 hours.
  Salmon-bread = 152 mg. per kilo.
  Proline + egg albumin = 116 mg. per kilo.
  Tryptophane + egg albumin = 105 mg. per kilo.

The cholagogue action (Table 65) is very well marked and the drop in bile salt concentration is well shown. This shows complete dissociation of bile salt output and cholagogue action.

Table 66 gives experimental data to show that tryptophane does not supplement egg albumin so as to increase the bile salt output. The bile salt output is lower with egg albumin plus tryptophane than during an earlier period on egg albumin alone. This is wholly different from the observations in similar experi-

ments given in Table 64. This difference we cannot explain at present. This dog was in good condition and the bile was sterile. The salmon-bread level for bile salt output (Table 66) is quite low (88 mg. per kilo) and is to be contrasted with the unusually high values of Table 64 (152 mg. per kilo).

TABLE 65.

Proline, Tryptophane, and Isatin in Diet.

Dog 28-87.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
3–1	11.3	Egg alb. 110, sugar 150, isatin 5				
2	11.4	" " 110, " 150, " 5	174	0.17	29	1060
3	10.5	" " 110, " 150, " 5	112	0.15	17	625
·4	10.5	" " 110, " 150, " 5	191	0.09	17	625
5	10.3	Salmon-bread 300	160	0.07	11	405
6	10.3	" 300	42	0.20	8	294
7	10.7	" 300	101	0.29	29	1060
8	10.5	" 300	107	0.37	39	1430
9	10.7	" 300	107	0.37	39	1430
10	10.3	" 300	85	0.35	30	1100
11	10.4	" 300	114	0.38	43	1580
12	10.7	Sugar 250	113	0.36	41	1500
13	10.7	" 250, proline 5, tryptophane 5	65	0.28	18	660
14	10.7	" 250, " 5, " 5	107	0.11	12	440
15	10.7	" 250, " 5, " 5	132	0.09	12	440
16	10.5	Salmon-bread 440	100	0.10	10	367
17	10.8	" 440	90	0.23	20	735
18	11.1		104	0.29	30	1100

Bile fistula operation December 20.
Bile infected, but dog in good clinical condition.
Bile salt average per 24 hours.
  Salmon-bread = 114 mg. per kilo.
  Proline, tryptophane + sugar = 39 mg. per kilo.
  Isatin + egg albumin = 59 mg. per kilo.

Tryptophane again shows little capacity to supplement egg albumin (Table 67). The fore period on egg albumin plus sugar gives a bile salt level of 47 mg. per kilo per 24 hours. When we add 5 gm. of tryptophane to each day's egg albumin feeding, we note a trivial increase to 54 mg. of bile salt per kilo. This

slight increase appears almost wholly on the 1st day of trypto-
phane feeding as though some material stored in the body was
pulled out and utilized.   This level is not sustained so we must
conclude that the evidence is two experiments against one that

TABLE 66.

Tryptophane and Egg Albumin Feeding.

Dog 29-169.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
3–9		Salmon-bread 255				
10	12.8	" 210	76	0.50	38	1400
11	12.8	" 135	58	0.45	26	950
12	12.4	Egg albumin 110, sugar 150	69	0.43	30	1100
13	12.6	" " 110, " 150	56	0.47	26	950
14	12.7	" " 110, " 150	52	0.43	22	810
15	12.6	" " 110, " 150	39	0.52	20	730
16	12.9	Salmon-bread 440	23	0.62	14	510
17	12.7	" 330	78	0.38	30	1100
18		" 210	72	0.52	37	1360
19	12.5	" 280	60	0.43	26	950
20	12.6	" 220	64	0.48	31	1140
21	12.5	Egg albumin 110, sugar 150	76	0.37	28	1030
22	12.3	" alb. 110, sugar 150, trypt. 5	26	0.60	15	550
23	12.3	" " 110, " 150, " 5	56	0.31	17	620
24	12.2	" " 110, " 150, " 5	·114	0.095	11	400
25	12.1	" albumin 110, sugar 150	114	0.11	13	480
26	12.1	" " 110, " 150	53	0.19	10	367
27	11.9	Salmon-bread 380	53	0.21	11	400
28	12.2		78	0.34	27	990

Bile fistula operation February 25.   Bile sterile throughout.
Bile salt average per 24 hours.
    Salmon-bread = 88 mg. per kilo.
    Egg albumin + sugar = 60 mg. per kilo.
    Tryptophane + egg albumin = 41 mg. per kilo.

tryptophane does not supplement egg albumin so as to increase
bile salt production.   The cholagogue effect (Table 67) is minimal
but it is observed that the flow is very low on egg albumin alone.
Also the output of bile salt on salmon-bread is below average,
87 mg. per kilo.

Glycine obviously supplements egg albumin and shows an increased bile salt output above the egg albumin base-line (Table 68). There are ample controls on the dog in Table 66 where the bile salt output per 24 hours is given for egg albumin plus

TABLE 67.

Tryptophane and Egg Albumin Feeding.

Dog 29-8.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
3–8		Salmon-bread 355				
9	16.4	" 355	88	0.39	34	1250
10	16.2	" 355	67	0.51	34	1250
11	16.3	" 355	68	0.61	41	1500
12	16.5	Egg albumin 110, sugar 150	105	0.40	42	1540
13	16.0	" " 110, " 150	56	0.54	30	1100
14	15.9	" " 110, " 150	12	0.71	8	294
15	15.7	" " 110, " 150	17	0.92	16	590
16	15.7	Salmon-bread 355	19	1.37	26	960
17	16.0	" 355	52	0.73	38	1390
18	16.0	" 355	74	0.61	45	1650
19	15.7	" 355	60	0.60	36	1320
20	16.2	" 355	74	0.50	37	1360
21	16.2	Egg albumin 110, sugar 150	65	0.60	39	1430
22	15.8	Egg alb. 110, sugar 150, trypt. 5	20	0.68	14	510
23	15.6	" " 110, " 150, " 5	51	0.54	28	1030
24	15.4	" " 110, " 150, " 5	43	0.46	20	730
25	15.3	" albumin 110, sugar 150	44	0.46	20	730
26	15.2	" " 110, " 150	18	0.97	17	624
27	15.2		13	1.18	15	550

Bile fistula operation February 20.   Bile sterile throughout.
Bile salt average per 24 hours.
  Salmon-bread = 87 mg. per kilo.
  Egg albumin + sugar = 47 mg. per kilo.
  Tryptophane + egg albumin = 54 mg. per kilo.

sugar and for egg albumin plus tryptophane. The average output is about 50 mg. per kilo, a little higher during the egg albumin feeding than subsequently when tryptophane was added. The general average of 50 mg. of bile salt per kilo per 24 hours

contrasts with this experiment (Table 68) where glycine added to the egg albumin lifts the bile salt output to 84 mg. per kilo. An identical experiment is given in Table 69 where the glycine

TABLE 68.

Glycine, Tryptophane, and Egg Albumin Feeding.

Dog ·29-169.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
5–10		Salmon-bread 387				
11	13.3	"　　360	122	0.33	41	1510
12	11.1	"　　360	121	0.33	40	1470
13	12.4	Egg alb. 110, sugar 170, glycine 20	94	0.36	34	1250
14	12.1	"　"　110,　"　170,　"　20	62	0.48	30	1100
15	12.0	"　"　110,　"　170,　"　20	42	0.47	20	730
16	12.0	"　"　110,　"　170,　"　20	52	0.47	24	880
17	12.0	Salmon-bread 440	70	0.52	36	1320
18	12.3	"　　440	84	0.36	30	1100
19	12.6	"　　280	96	0.42	40	1470
20	12.2		82	0.44	36	1320
6–4		Salmon-bread 310				
5	12.3	"　　210	87	0.39	34	1250
6	12.3	Sugar 150	87	0.44	38	1400
7	11.8	Cane-sugar 150, glycine 20, trypt. 5	18	0.41	7	260
8	11.4	"　　150,　"　20,　"　5	57	0.43	25	920
9	11.2	"　　150,　"　20,　"　5	54	0.28	15	550
10	11.2	Salmon-bread 280	21	0.37	8	290·
11	11.5	"　　255	64	0.45	29	1060
12	11.8		84	0.43	36	1320

Bile fistula operation February 25.　Bile sterile throughout.
Bile salt average per 24 hours.
　Salmon-bread = 107 mg. per kilo.
　Glycine + egg albumin = 84 mg. per kilo.
　Glycine, tryptophane + sugar = 52 mg. per kilo.

plus egg albumin diet shows a bile salt output of 87 mg. per kilo. For control of Table 69, see the egg albumin experiments in Table 67 where the egg albumin feeding shows the usual bile salt output and the negative effect of tryptophane.

Glycine evidently supplies an amino acid known to be missing in egg albumin, which increases the bile salt output, but this level

TABLE 69.

Glycine, Tryptophane, and Egg Albumin Feeding.

Dog 29-8.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
5–10		Salmon-bread 440				
11	15.7	" 440	92	0.49	45	1650
12	15.7	" 440	93	0.48	45	1650
13	16.0	Egg alb. 110, sugar 170, glycine 20	66	0.57	38	1400
14	15.6	" " 110, " 170, " 20	57	0.62	35	1290
15	15.4	" " 110, " 170, " 20	58	0.64	37	1360
16	15.3	" " 110, " 170, " 20	50	0.77	39	1430
17	15.3	Salmon-bread 440	30	1.01	30	1100
18	15.6	" 440	48	0.83	40	1470
19	15.7	" 400	74	0.64	47	1730
20	15.7		74	0.57	42	1540
6–3		Salmon-bread 440				
4	16.0	" 440	96	0.53	51	1870
5	16.0	" 440	77	0.53	42	1540
6	16.0	Sugar 150	84	0.56	47	1730
7	15.3	Cane-sugar 150, glycine 20, trypt. 5	36	0.63	23	840
8	15.0	" 150, " 20, " 5	60	0.46	28	1030
9	14.7	" 150, " 20, " 5	56	0.44	25	920
10	14.7	Salmon-bread 440	34	0.52	18	660
11	15.3	" 440	37	0.68	25	920
12	15.7		64	0.61	39	.1430

Bile fistula operation February 20.   Bile sterile until May 20.

Bile salt average per 24 hours.

Salmon-bread = 97 mg. per kilo.

Glycine + egg albumin = 87 mg. per kilo.

Glycine, tryptophane + sugar = 59 mg. per kilo.

is below that for salmon-bread feeding and far below the high levels due to the feeding of meat products.

Cholagogue effect is missing in these glycine experiments (Tables 68 and 69), and differentiates sharply this amino acid from tryptophane and proline.

Glycine and tryptophane fed with sugar show about the same bile salt output as would be expected with tryptophane and sugar alone. There is a temporary rise in the 1st and less in the 2nd day of feeding but it is not sustained and suggests the removal of an unknown material stored in the body to supplement these amino acids (Tables 68 and 69). We may compare these slight increases in bile salt due to tryptophane and glycine—52 mg. per kilo and 59 mg. per kilo with the usual level due to sugar feeding alone—35 mg. of bile salt per kilo.

Cholagogue action with tryptophane and sugar is very conspicuous (Table 65 above and Table 32 in Paper III (2)). When *glycine* is added we note almost complete obliteration of this cholagogue effect. This observation suggests that glycine in some way *inhibits* the cholagogue action of tryptophane. It will be interesting to determine whether this inhibition may act on other cholagogues.

<div align="center">DISCUSSION.</div>

Glycine is an interesting amino acid in relation to bile salt metabolism. It is generally accepted that it can be formed in the body. Nevertheless we felt that its presence in abundance within the body might facilitate certain amino acid linkages which we assume must take place in the liver or elsewhere as the body produces bile salt from day to day. Glycine evidently supplements egg albumin and increases the bile salt output. If we assume that this reaction is due to the absence of glycine in the make-up of egg albumin, it shows that the body can *use* glycine more readily for bile salt production than it can *produce* it for this purpose.

Glycine shows no cholagogue effect and in this respect differs sharply from tryptophane and proline. In fact the evidence suggests that glycine may inhibit the strong cholagogue effect of tryptophane.

Glycine, tryptophane, and sugar feeding (Tables 68 and 69) show more bile salt output than we note with tryptophane and sugar alone. We may believe that the glycine facilitates certain amino acid linkages to give this result but the reaction is not sustained and suggests the withdrawal of unknown substances stored in the body, giving only a temporary rise.

SUMMARY.

Proline in certain respects resembles tryptophane when used to supplement certain diets unfavorable for bile salt output in bile fistula dogs.

Proline added to the salmon-bread diet may increase the bile salt output per 24 hours by about 50 per cent. There is also a definite cholagogue effect.

Proline added to the egg albumin diet may increase the bile salt output per 24 hours by as much as 100 per cent. There is a definite cholagogue effect.

Proline and tryptophane with sugar give practically the same bile salt reaction to be expected from sugar alone. Some other substance is needed to supplement these amino acids to complete the construction cycle of bile salt in the body.

Tryptophane supplements gelatin in extraordinary fashion and causes a notable rise from the gelatin diet level of 70 mg. per kilo to 180 mg. of bile salt per kilo per 24 hours.

Tryptophane does not supplement egg albumin (two experiments out of three) and causes no increase or only a slight increase in bile salt output.

Tryptophane always shows some cholagogue effect which may be very conspicuous. It is suggested that the *pyrrole ring* in proline and tryptophane may be responsible for the cholagogue effect.

Glycine supplements egg albumin in the diet, and increases the output of bile salts in moderate fashion.

Glycine gives no cholagogue effect and differs sharply from proline and tryptophane. In fact it may actually inhibit the cholagogue effect so conspicuous with tryptophane alone.

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BILE SALT METABOLISM.

VII. INDENE, HYDRINDENE, AND ISATIN.

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It is well known that taurocholic acid makes up the total bile acid content of dog bile. It has been shown (1) that the body can supply taurine in almost any amount as may be needed to combine with the cholic acid to complete the bile salt production. It is highly probable that most of this taurine comes from cystine. The source of *cholic acid* therefore holds the main interest in any study of bile acid metabolism as cholic acid is the limiting factor in bile salt output by the bile fistula dog.

The structural formula of cholic acid has been established by Wieland and his pupils as described in a previous paper (2). Cholic acid contains two *indene rings* (combined 6-carbon and 5-carbon rings). Our interest in the indene ring and related derivatives needs no further comment. Can the body build up the indene ring in its internal synthetic metabolism? If so, what substances may contribute to this end?

Experimental Observations.

Indene and its derivatives are difficult to prepare in pure form and these chemicals have been supplied by the Laboratory of Synthetic Chemistry of the Eastman Kodak Company.[1] The indene used was listed as Practical Grade and therefore contained impurities some of which may be toxic. It causes much vomiting in dogs when given by stomach tube and this digestive upset is responsible for unsatisfactory results. We assume that the cholagogue effect is due to the indene and the evidence points

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[1] We are greatly indebted to Dr. Hans Clarke and Mr. W. W. Hartman for helpful advice and friendly cooperation.

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his way; however we hope soon to have a supply of pure indene to stablish this point beyond a doubt.

The hydrindene dicarboxylic acid was given in pure form and his also is somewhat toxic but not to the same extent as indene. It was supplied in the form of white crystals. Its formula is given as

$$\text{hydrindene} \begin{array}{c} -CH_2 \\ -CH_2 \end{array}\!\!\!\!\!> C(COOH)_2$$
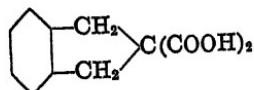
TABLE 71.

Indene Added to Salmon-Bread Diet.

Dog 28-9.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
10–11		Salmon-bread 440	150	0.28	42	1540
12	13.2	" 440	114	0.34	39	1430
13	13.2	" 380 + indene 5 cc.	270	0.19	52	1910
14	13.3	" 380 + " 5 "	256	0.18	46	1690
15	12.9	" 320 + " 5 "	325	0.13	42	1540 ·
16	13.0	" 320 + " 5 "	354	0.06	21	770
17	13.2	" 380 + " 5 "	290	0.05	15	550
18	12.4	" 45	199	0.08	16	590
19	12.7	" 440	192	0.20	38	1400
20	12.7	" 440	160	0.30	48	1760
21	12.7					

Bile fistula operation September 21.
Considerable vomiting due to indene.
Bile salt average per 24 hours.
  Salmon-bread = 104 mg. per kilo.
  Indene + salmon-bread = 100 mg. per kilo.

Table 71 shows the most satisfactory of several experiments in which indene was given by stomach tube. This caused vomiting about every day after the drug was given but in spite of this the dog ate the salmon-bread in satisfactory amounts. The definite fall in bile salt output during the last 2 days is noted and probably must be explained by some intoxication. The average bile salt output per 24 hours is about the same for the indene period and control periods.

Cholagogue'effect is conspicuous and reaches a high level attained in other experiments only by bile salt feeding.   We may say that other experiments show the same effect in other dogs but these dogs did not tolerate the drug as well and stopped eating after a day or 2.   It did not seem worth while to tabulate these data but we hope

TABLE 72.

Hydrindene Dicarboxylic Acid, Chloroform by Mouth.

Dog 29-8.

Date.	Wt.	Diet.		Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
					In 1 cc. bile.	Total output.	
	kg.		*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
6–14		Salmon-bread	440				
15	15.5	" 440		99	0.65	64	2350
16	15.5	Sugar 150, hydrindene 3		77	0.54	42	1540
17	15.0	" 150, " 3		44	0.69	30	1100
18	14.7	" 150, " 3		36	0.65	23	840
19	14.4	Salmon-bread 440		30	0.68	20	730
20	14.9	" 440		54	0.64	35	1290
21	15.2	" 440		102	0.51	52	1910
22	15.1	" 440		118	0.46	54	1980
23	15.0	" 440, chloroform 3 cc.		92	0.49	45	1650
24	15.0	" 440, " 3 "		56	0.56	31	1140
25	15.3	" 440		11	0.37	4	147
26	15.2	" 440		12	0.20	2	73
27	15.0	" 440		60	0.36	22	810
28	15.0	" 440		106	0.40	42	1540
29	15.2			142	0.41	58	2130

Bile fistula operation February 20.
Bile contained *Bacillus subtilis;* dog in good clinical condition.
Chloroform by mouth in starch solution.
Bile salt average per 24 hours.
  Salmon-bread = 100 mg. per kilo.
  Hydrindene = 61 mg. per kilo.

to do further work with other derivatives and highly purified indene. So far the evidence indicates that the dog cannot utilize indene to build up cholic acid in the body but the indene ring seems to have a very powerful cholagogue effect, almost the equivalent of cholic acid itself.   It cannot be denied that reduction products of indene present in this material may be responsible.

We had high hopes that hydrindene dicarboxylic ácid might be non-toxic and further, might influence bile salt output. We were disappointed on both counts. 3 gm. is about as much of this chemical as the dog can tolerate and even this amount may cause vomiting. Table 72 shows that hydrindene given with sugar causes no notable change other than what would be expected from sugar alone (compare with Table 69, an experiment of similar type on the same dog with sugar and amino acids). The bile salt output during this period amounts to 61 mg. per kilo which is not quite down to the sugar level but it shows a progressive fall and we may explain this slight increase as due to some materials previously stored in the body and released in this emergency. There is no cholagogue effect after hydrindene feeding in striking contrast to indene and we may assume that the added radicals are responsible for this loss of physiological potency.

We may refer to a second experiment with hydrindene dicarboxylic acid (Table 69, Paper VI). This dog shows a reaction to sugar and hydrindene which is identical to that expected from sugar alone. The sugar level of bile salt output is attained with sugar and hydrindene feeding and there is no cholagogue effect. The evidence is that under such conditions hydrindene is inert as regards bile salt output. Table 72 also gives a chloroform experiment which is discussed in the next paper.

Isatin and *indigo* are alike in that isatin contains one indole ring and indigo contains two indole rings. Tryptophane contains an indole ring and its interesting capacity to supplement certain food substances and cause a high bile salt output has been discussed in the preceding paper (Paper VI). We have reported previously (2) other experiments with isatin and indigo to show that under certain conditions these chemicals might cause a moderate increase in the output of bile salts.

Table 73 shows the effect of isatin alone and isatin plus indigo added to a small amount of salmon-bread. The isatin and indigo were added to the food and eaten without trouble except on 1 day as noted. At first sight the reaction seems negative as the average output for all these three periods is practically identical: 122, 129, and 131 mg. per kilo. But on an intake of 110 gm. of salmon-bread alone this dog would show an output of bile salt probably below 100 mg. per kilo. We believe therefore the indigo and isatin

have supplemented somewhat the bile salt output due to salmon-bread alone.   The casein experiment (Table 73) has been discussed above (Paper V).

TABLE 73.

Isatin, Indigo, and Casein.

Dog 28-46.

Date.	Wt.	Diet.			Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
						In 1 cc. bile.	Total output.	
	kg.		*gm.*		*cc.*	*mg.*	*mg.*	*mg.*
12–28		Salmon-bread 110, isatin 4						
29	11.5	"	110, " 4		168	0.23	39	1430
30	11.5	"	110, " 4		166	0.23	38	1400
31	11.2	"	110, " 4, indigo 4		162	0.24	39	1430
1–1	11.6	Refused food			161	0.23	37	1360
2	11.0	Salmon-bread 110, isatin 4, indigo 4			126	0.33	42	1540
3	11.0	"	110, " 4, " 4		118	0.32	38	1400
4	11.1	"	220		145	0.28	41	1510
5	11.3	"	330		140	0.31	43	1580
6	11.4	"	365		160	0.27	43	1580
7	11.5	"	220		151			
8	11.1	"	190		108	0.35	38	1400
9	10.9	"	375		101	0.38	38	1400
10	11.0	"	210		105	0.37	39	1430
11	10.7	Casein 175, sugar 125			112	0.32	36	1320
12	11.0	"	175, " 125		113	0.40	45	1650
13	11.2	"	175, " 125		146	0.47	69	2530
14	11.2	"	115, " 82		124	0.46	57	2090
15	10.9				80	0.63	50	1830

Bile fistula operation, December 11.
Bile contained bacteria; but dog in good clinical condition.
Bile salt average per 24 hours.
  Salmon-bread = 131 mg. per kilo.
  Isatin + salmon-bread = 122 mg. per kilo.
  Isatin + indigo + salmon-bread = 129 mg. per kilo.
  Casein + sugar = 182 mg. per kilo.

A second experiment with isatin is given in Table 65 (Paper VI). In this dog the isatin was used to supplement an egg albumin-sugar diet.   It is frankly negative as regards isatin and the bile salt

output.   The high figure of the 1st day is evidence of a reserve storage and has no significance as regards the isatin.

Cholagogue effect is distinct when we examine the isatin experiments both with salmon-bread and egg albumin.   The flow of bile on egg albumin and sugar diet is about 50 to 60 cc. (Table 52, Paper V) which is to be compared with the isatin-albumin experiment (Table 65, Paper VI) where the total bile flow per 24 hours is 160 cc.

DISCUSSION.

We feel that the *cholagogue action* of various substances holds much of interest for the student of bile physiology.   Bile salt stands at the head of the list but we recall the striking effect of *indene* in causing maximal bile flow in spite of gastrointestinal irritation which tends to inhibit bile flow.

Tryptophane and isatin show a notable cholagogue effect under practically all conditions.   Proline also shows a distinct cholagogue effect and by contrast glycine, *p*-hydroxyphenylglycine, and tyrosine are practically inert.

The above facts suggest as responsible factors for the cholagogue stimulus to the hepatic epithelium the indole ring, the indene ring, and the pyrrole ring.   It is possible that we may find that the pyrrole ring and the 5-carbon ring of indene are largely responsible, as the benzene ring alone is apparently inert.

This suggests some physiological relationship between the indole and indene rings which is of added interest in view of the great increase of bile salt (indene rings) when tryptophane (indole ring) is given to the bile fistula dog on a gelatin diet.

The evidence coming from a study of amino acids (Papers III and VI) and from a study of certain ring compounds given above (indene and isatin) is strong that the body is able to synthesize the indene ring but it does not appear able to utilize indene as such when given by stomach.   Cyclic amino acids probably are in part concerned with the amino acid grouping effected in the liver cell (Paper VIII) which leads to the production of bile salts.

SUMMARY.

Indene given by mouth to a bile fistula dog does not supplement certain diets and does not increase the output of bile salts.   The

body does not utilize indene to synthesize the bile salt containing two indene rings, at least under the conditions of these experiments.

Hydrindene dicarboxylic acid under similar conditions is completely inert.

Isatin given by mouth may increase the bile salt output under certain conditions.

Indene especially and also isatin have a *powerful cholagogue effect.*

Substances having definite cholagogue action contain the *indene* ring (bile salts) or the *indole* ring (isatin and tryptophane) or the *pyrrole* ring (proline). It is suggested that these ring forms are responsible for the cholagogue effect upon the liver cell. Various possibilities are discussed. It would seem that the body is capable of synthesizing the indene ring.

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BILE SALT METABOLISM.

VIII. LIVER INJURY AND LIVER STIMULATION.

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The experiments tabulated below may seem wholly unrelated but in each experiment substances are given which under certain conditions exert some influence upon the liver cell. The relation of bile salts to the hepatic cell function is of profound importance from two points of view. The liver cell is probably wholly responsible for the production of bile salts and the internal part of the bile salt cycle has an important influence upon the function of the hepatic cell. For example chloroform poisoning shows the startling effect of slight liver injury on bile salt output. Long continued observations with bile fistulas show a disturbed physiology undoubtedly related to the lack of the normal internal circulation of the bile salts.

The experiments with liver fractions and ferric citrate call for some explanation. The liver fractions were standard.preparations known to be potent, one in pernicious anemia and the other in secondary anemia, prepared by methods described elsewhere (1, 2). Ferric citrate is known to be potent in causing much hemoglobin regeneration in experimental anemia in dogs. Evidence to be published from this laboratory indicates that the liver plays an important function in the preliminary grouping of amino acids to form the precursors of hemoglobin which are utilized by the marrow cells in turning out the finished red blood cells into the circulation. If our interpretation of these experiments is correct any materials which favor new hemoglobin production might affect in some way the internal metabolism of the liver cell as it is probable that these liver cells may use amino acids for one purpose under certain circumstances, and

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for a totally different purpose under other conditions.   There may be *intermediates* which are fluid assets capable of use according to the demands of the moment.

For these reasons we tested these substances which favor new hemoglobin building in anemic dogs to ascertain whether the bile salt output was modified.   We see no change in the normal dogs but in the Eck fistula (Table 92, Paper IX) we see a fall in bile salts.   It is important also to study the effect in anemic bile fistula dogs as some of the amino acids may be diverted from bile salt to hemoglobin production.

Experimental Observations.

The influence of mild chloroform poisoning upon bile salt secretion was studied in detail by Smyth and Whipple (3).   They used the familiar open fistula and shorter periods of bile collection. They pointed out that very small amounts of chloroform by mouth might cause a marked depression in bile salt output, yet if the dog was killed after 2 or 3 days no histological evidence of liver injury was detectable.   That the liver is very sensitive to chloroform by mouth or by inhalation is well known and this highly specific effect of chloroform on the hepatic epithelium is of much experimental value.   This type of evidence gives strong support to the argument that the hepatic cell is the source of bile salt production in the body.

The experiments given in Tables 81 and 82 and in Table 72, Paper VII, show that these closed bile fistulas with complete 24 hour collections show identical results and great bile salt decrease due to minor liver injury from chloroform.   This obtains whether the bile is sterile or infected, whether the chloroform is given by mouth or by inhalation.   We believe the evidence points to the decreased bile salt output as due to *depressed functional activity* of the hepatic epithelium, not to obstruction of outflow or mechanical influence due to injured liver cells.

Table 81 shows a satisfactory experiment with chloroform anesthesia in which the depressed bile salt output is obvious at a glance.   This depression is maximal on the 2nd and 3rd days when chloroform injury is most apparent if the liver is examined. The preceding fasting 24 hours makes the dog more susceptible to chloroform and gives more uniform injury.   From experience

with a great many chloroform experiments we feel that this dog showed a minimal histological injury with a few necrotic liver cells in the centers of the lobules. There was not the slightest clinical indisposition and the dog ate well throughout. Such injuries are repaired promptly within 5 to 7 days and we note a return to normal bile salt output in that time.

This same dog (Dog 29-8, Table 72, Paper VII) shows an identical reaction due to 3 cc. of chloroform by mouth given on 2 successive days. In this experiment we note the same maximal

TABLE 81.

Chloroform Anesthesia Causes Fall in Bile Salt Output.

Dog 29-8.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
6–29		Salmon-bread 440				
30	15.3	Fasting	144	0.40	58	2130
7–1	14.6	Salmon-bread 440, chloroform	88	0.43	38	1400
2	14.8	" 440	50	0.36	18	660
3	15.0	" 440	14	0.20	3	110
4	15.0	" 440	25	0.19	5	184
5	14.9	" 440	58	0.28	16	590
6	15.1	" 440	84	0.32	27	990
7	15.3	" 440	103	0.38	39	1430
8	15.4		98	0.42	41	1500

Bile fistula operation February 20.   Bile sterile.

Chloroform anesthesia for 1 hour.   No subsequent clinical disturbance.

drop on the 2nd day and prompt return to normal, presumably as the slight liver cell injury is repaired. The amount of actual liver cell damage was probably less in this experiment than in the preceding one where the chloroform was given during 1 hour of anesthesia.

Table 82 shows a chloroform experiment where the drug is given by stomach tube; 3 cc. on successive days. The result is much like that noted above (Table 72, Paper VII) but the experiment is not quite as satisfactory. The drop in bile salt output is conspicuous enough but there was slight clinical disturbance and

loss of appetite not present in the two experiments given above. At the end of the experiment (June 29, not shown in Table 82) the tube became kinked, with a day of bile obstruction which confused the picture.   This dog was killed with ether on July 7 which was 9 days after completion of observations in Table 82. Autopsy showed evidence of slight liver injury and possibly a little central necrosis due to the chloroform given by mouth.

TABLE 82.

Chloroform Given by Mouth.

Dog 29-169.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
6–14		Salmon-bread 250				
15	11.8	" 170	139	0.43	60	2200
16	11.6	Sugar 150, hydrindene 3	112	0.30	34	1250
17	11.5	" 150, " 3	29	0.64	19	700
18	11.4	" 150, " 3	15	0.60	9	330
19	11.4	Salmon bread 360	19	0.61	12	440
20	11.8	" 300	7	0.73	5	184
21	11.8	" 440	135	0.26	35	1290
22	11.9	" 440	164	0.28	46	1700
23	11.7	" 200, chloroform 3 cc.	148	0.32	48	1760
24	11.5	" 230, " 3 "	106	0.24	25	920
25	11.5	" 145	18	0.10	2	73
26	11.4	" 145	13	0.05	1	37
27	11.3	" 22	10	0.08	1	37
28	11.4		15	0.23	3	110

Bile fistula operation February 25.
Bile contained *Bacillus subtilis*; but dog was in good clinical condition.

The time interval between the chloroform injury and autopsy makes it impossible to speak with certainty on these points. The hydrindene experiment has been discussed in Paper VII.

Table 83 shows experiments with liver fractions known to be potent in human pernicious anemia and in experimental anemia in dogs.   The dog was used for this series of experiments very shortly after the bile fistula was produced.   The bile was sterile throughout, the dog was in excellent condition, and actually

gained in weight during this period.   This may have been due in part to the liver fractions as we have observed on many occa-

TABLE 83.

Liver Fractions Potent in Anemia Added to Salmon-Bread.

Dog 28-177.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
5–17		Salmon-bread 440				
18	13.8	" 440	170	0.35	60	2200
19	13.8	" 440	209	0.41	86	3160
20	13.9	" 440	194	0.36	70	2570
21	14.0	Liver fraction (P.A.)*	178	0.32	57	2090
22	13.7	" " " *	165	0.31	51	1870
23	14.0	" " " *	179	0.29	52	1910
24	14.2	" " " *	205	0.35	72	2640
25	15.2	Salmon-bread 440	200	0.36	72	2640
26	14.5	" 440	184	0.32	59	2160
27	14.4	" 440	161	0.22	35	1290
28	14.7	" 440	194	0.32	62	2280
29	14.9	Liver fraction (2nd anemia)†	188	0.38	71	2610
30	15.0	" " " " †	220	0.34	75	2750
31	14.9	" " " " †	128	0.32	41	1500
6–1	15.0	" " " " †	124	0.36	45	1650
2	14.9	Salmon-bread 440	138	0.34	47	1730
3	14.6	" 440	190	0.37	70	2570
4	14.4		158	0.38	60	2200

Bile fistula operation May 14.   Bile sterile throughout.

Bile salt average per 24 hours.

  Salmon-bread = 162 mg. per kilo.

  Liver fraction (pernicious anemia) = 158 mg. per kilo.

  Liver fraction (secondary anemia) = 128 mg. per kilo.

  * Daily diet salmon-bread 400, salmon 100, liver fraction (pernicious anemia) 600 equivalent.

  † Daily diet salmon-bread 400, salmon 40, liver fraction (secondary anemia) 600 equivalent.

sions (4) that liver feeding has a beneficial effect upon bile fistulas. The very high level of bile salt output is to be explained in part by the large food intake and excellent clinical condition but to a certain degree by the short interval elapsing between the bile

fistula operation and the experimental period. We have observed in a considerable series of dogs that the base-line output on salmon-bread has a tendency to fall a little from month to month after the operation even when all conditions are uniform, the bile remaining sterile and appetite good. This is probably a slow change due to continued deprivation of bile salts which in the normal internal cycle influence the function of the liver cells. We hope to report further on this point.

TABLE 84.

Ferric Citrate Added to Standard Diet.

Dog 29-8.

Date.	Wt.	Diet.			Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
						In 1 cc. bile.	Total output.	
	kg.		gm.		cc.	mg.	mg.	mg.
5-23		Salmon-bread 440						
24	15.7	"	440		115	0.43	50	1840
25	15.7	"	440		140	0.38	53	1950
26	15.6	"	440 + ferric citrate 0.44		144	0.39	56	2060
27	15.6	"	440 + " " 0.44		139	0.37	52	1910
28	15.9	"	440 + " " 0.44		148	0.38	56	2060
29	15.9	"	440 + " " 0.44		140	0.38	53	1950
30	15.7	"	440		122	0.42	51	1870
31	15.8	"	440		106	0.43	46	1690
6-1	15.6	"	440		102	0.43	44	1620
2	15.7	"	440		76	0.50	38	1400
3	15.9				68	0.60	41	1510

Bile fistula operation February 20. Bile sterile.
Bile salt average per 24 hours.
  Salmon-bread = 109 mg. per kilo.
  Salmon-bread + iron salt = 116 mg. per kilo.

Table 83 shows that these liver fractions have no conspicuous immediate effect on the bile salt output. The liver fraction potent in human pernicious anemia was given daily in liberal doses of 600 gm. equivalent—that is, the material from 600 gm. of fresh liver. This product (No. 343) was prepared by Eli Lilly and Company and tested and standardized on human pernicious anemia as described elsewhere (1).

The liver fraction potent in dogs in experimental anemia due to bleeding was likewise given in 600 gm. equivalents each day mixed with the food. We note a slight decrease in the bile salt output but we hesitate to place emphasis on this single observation. We hope to make more observations on normal and anemic bile fistula dogs with other diets including these and other liver fractions. It appears that these liver fractions like meat products in general tend to increase somewhat the flow of whole bile.

Table 84 shows the negative influence of large doses of ferric citrate added to the salmon-bread ration. This dog was not anemic and presented a high normal hemoglobin of 146 per cent. As we believe the liver cell plays a significant rôle in the construction of new hemoglobin in the body, it is obvious that iron might influence the metabolism of the liver cell and this might be reflected in a change in bile salt output. In this dog the output on salmon-bread is 109 mg. of bile salt per 24 hours per kilo of body weight. With iron citrate we note a bile salt output of 116 mg. per kilo. We may refer to a different reaction to iron feeding in an Eck fistula dog (Table 92) which is discussed in Paper IX.

We wish to refer to an experiment in Table 62, Paper VI, which is closely related to the ferric citrate experiment. *Apricots* are potent in influencing new hemoglobin regeneration in experimental anemia and the potency resides in the apricot ash; in other words in inorganic salts, among which iron is present. This apricot experiment is identical with the ferric citrate experiment (Table 84). It will be of interest to repeat these same experiments in anemic bile fistula dogs to find whether the reaction remains negative.

Table 85 gives information on the influence of continued bile refeeding. Bile salt gives a strong stimulus to the liver cell and as the salt circulates normally in the body it is continually being absorbed from the intestine and secreted by the liver epithelium. This normal cycle when interrupted by a bile fistula causes slight disturbances within the body and as the weeks go by we note a variety of abnormalities—loss of weight and appetite, loss of bone substance which may in some cases lead to fracture, duodenal ulcers, and abnormal bleeding from the mucous membranes, or fistula. Some of these abnormalities may be checked by liver feeding and by other diet measures and by bile feeding. Relating to these abnormalities *bile salt* is the most important single constit-

TABLE 85.

Bile Salt Output Levels with Bile Refeeding.

Dog 28-9.

Date.	Wt.	Diet.		Bile fed.		Bile secreted.			
				Vol.	Total tauro-cholic acid.	Vol.	Amino nitrogen.		Total tauro-cholic acid.
							In 1 cc. bile.	Total output.	
	kg.	*gm.*		*cc.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*
10–29		Salmon-bread 440		300		132			
30		"	440	300		223	0.75	170	6200
31	12.5	"	440	399		312	0.80	250	9200
11–1	12.4	"	440	460		360	0.77	278	10200
2	12.8	"	440	384	11600	384	0.83	317	11600
3	12.7	"	440	413	12700	413	0.84	346	12700
4	13.0	"	440	410	13600	410	0.90	370	13600
5	12.8	"	385	457	13800	457	0.80	375	13800
6	12.7	"	440	371	11200	371	0.83	305	11200
7	12.8	"	440	358	11200	358	0.85	305	11200
8	12.8	"	440	403	11000	403	0.74	300	11000
9	12.7	Beefsteak 400		0		412			
10	12.6	"	400	450		216	0.80	173	6300
11	12.7	"	400	420	13800	420	0.89	375	13800
12	12.4	"	530	382	14700	382	1.05	400	14700
13	12.4	"	530	452	15000	452	0.90	407	15000
14	12.6	"	510	433	16400	433	1.03	445	16400
15	12.3	"	430	461	18200	461	1.07	495	18200
16	12.6	"	380	464	18400	464	1.08	500	18400
17	12.5	"	390	408	16400	408	1.09	445	16400
18	12.3	Salmon-bread 440		448	16900	448	1.03	460	16900
19	12.5	"	440	400	16500	400	1.12	450	16500
20	11.3	"	440	389	16700	389	1.17	456	16700
21	12.8	"	440	420	16000	420	1.04	437	16000
22	12.6	"	440	459	14800	459	0.88	404	14800
23	12.9	"	440*	600	21700	475	0.91	432	15900
24	13.0	"	440	577	20200	577	0.96	550	20200
25	12.9	"	440	537	19100	537	0.97	520	19100
26	13.0	"	440	496	16900	496	0.93	460	16900
27	12.8	"	440	490	17300	490	0.96	470	17300
28	13.1			492	16500	492	0.92	450	16500

Bile fistula operation September 21.   Dog in excellent clinical condition.
* Bile salt, 5.8 gm., added to usual refeeding on November 23.

uent of the whole bile.   Some of our bile fistula animals get bile by mouth daily.   We have reported elsewhere (5) some observations on bile refeeding to show the amount of bile which might be kept in circulation within the body.

Table 85 gives more information about the high levels of bile salt circulation effected by bile refeeding.   This dog was in excellent clinical condition throughout the entire experiment and shows a slight gain in weight.   The bile feeding (Table 85) was begun before the first date (October 29) to save time and analyses.   The bile was fed back each day by stomach tube after the bile collection.   The amount used for analysis was replaced by stock bile which may introduce an error of a few mg. of bile salt but this is negligible in these large output figures.   We note a steady rise to a maximum of 13,800 mg. of bile salt per 24 hours on the salmon-bread diet.   Then there is a slight fall to about 11,000 mg. of bile salt.

When *meat feeding* begins we note a sharp rise to *higher levels,* a maximum of 18,400 mg. of bile salt per 24 hours or an output per kilo of 1472 mg.   This figure is about 7 times as great as the average output of bile salt per 24 hours when beef is given and bile is excluded from the intestine.   From this high maximum there is a slight fall to the sustained level of 16,500 mg. per 24 hours.   The dog now given a salmon-bread diet shows a slow fall toward the salmon-bread level but this is not reached within the period of observation.

A large *added dose of whole bile* was given with the usual refeeding on November 23, Table 85.   The added bile salt was 5.8 gm., giving a total intake for that day of 21.7 gm.   The next day shows an output of 20.2 gm. and with daily refeeding we note a steady fall of 1 to 2 gm. per day till the level of 16.5 gm. is reached at the end of the experiment.

The older experiments (5) showed a maximum plateau level of bile excretion on salmon-bread diet with bile refeeding of 700 to 800 mg. of bile salt per kilo per 24 hours.   The dog given in Table 85 shows a plateau level on salmon-bread of about 875 mg. of bile salt per kilo.   With beef muscle feeding this plateau level is pushed up much higher to a maximum of 1472 mg. of bile salt per kilo. We believe this is due in part to an actual stimulus acting on the liver epithelium.   As in other experiments (5) we note that large

supplementary bile feeding will push up the bile salt output but it falls back again when the supplementary feeding is discontinued. This fall back to the plateau level for the given food intake requires several days.

At various times we have debated the *fate of the bile salt surplus* as it is formed and circulated in the body.   Obviously it is used up or discarded as it is being formed and continuously circulated in the normal dog.   Does this surplus escape by way of the urine or feces or is it destroyed in the body tissues?   Observations recorded below indicate that the *surplus does not escape* by way of the urine.

Dog 28-9, Table 85, was receiving large amounts of bile by refeeding and a large extra amount by supplementary feeding (5.8 gm. of bile salt November 23).   The urine was collected continuously from November 20 to 24.   The collections were made twice daily and the urine was kept in the ice box until analyzed.   A 24 hour collection taken on November 20 and also a similar collection taken on November 24 were analyzed by a method somewhat similar to that originally advocated by Schmidt and Merrill (6). In the present experiment the collection on November 20 consisted of 910 cc. of urine; that on November 24 consisted of 884 cc.   In each case 100 cc. of urine were evaporated to about 10 to 15 cc. 80 cc. of 95 per cent alcohol were added.   The mixture was heated and centrifuged.   The supernatant fluid was evaporated to dryness and the residue dissolved in about 10 cc. of distilled water.   Solid magnesium sulfate was added to saturation.   A yellowish precipitate formed, and this was recovered by centrifugalization.   The precipitate was washed with saturated magnesium sulfate and again recovered by centrifugalization.   To dissolve the bile salts from this precipitate several changes of absolute alcohol were used. This alcoholic extract was made up to 50 cc. with alcohol.   20 cc. portions of this were evaporated to dryness.   To ·one of these were added 10 cc. of 40 per cent sodium hydroxide, and hydrolysis was carried out in the manner regularly used by us in bile salt determinations.   A 3 cc. sample of this analyzed in the Van Slyke apparatus liberated 0.21 cc. of nitrogen.   The other alcoholic extract which had been evaporated to dryness was made up to 10 cc. with water and this was analyzed in the Van Slyke apparatus without further treatment.   In this case 0.18 cc. of nitrogen were

liberated.   The barometric pressure was 750 mg. and the temperature was 23°.   From these data one can estimate that 100 cc. of this urine contained not more than 5.1 mg. of bile salt.   On this basis the total 24 hour collection contained only 46 mg. of bile salt. Analysis was also made on the sample collected on November 24. The Van Slyke reading before hydrolysis was 0.22 cc. of gas; the reading after hydrolysis was 0.23.   One may conclude that the entire 24 hour collection of the urine contained not more than about 15 mg. of bile salt.

As a control for these experiments normal dog urine was mixed with a known amount of sodium taurocholate.   The mixture was subjected to the above analytic procedure and the results obtained are in very close agreement with those obtained when the sodium taurocholate was made up with water and analyzed without further treatment.   The differences did not exceed 4 per cent.

<div align="center">SUMMARY.</div>

Chloroform injury of the liver of slight grade will cause a great drop in bile salt output which returns to normal with the rapid repair of the liver cells.   If the dose is small there will be no clinical disturbance.   The actual injury of the liver cells is known to be trivial whether the chloroform is given by mouth or by inhalation. This indicates that the liver cell produces the bile salt.

Liver fractions of certain character and iron salts are known to influence hemoglobin regeneration and it is believed that the liver takes a part in fabricating new hemoglobin.   A bile fistula in a non-anemic dog shows no change in bile salt output when these substances are added to the control diet.

Daily refeeding of all bile secreted by a bile fistula dog on a *salmon-bread diet* may raise the bile salt secretion to a plateau level of 875 mg. per kilo per 24 hours.   On a *meat diet* this plateau level may be raised to a maximum of 1470 mg. of bile salt per kilo.   It seems reasonable to assume that this increase in the plateau level is due at least in part to a stimulus acting on the liver epithelium.

A bile surplus produced in the body or absorbed from the intestinal tract will gradually disappear from the bile salt circulation.   Evidence is given above to show that the surplus does not escape by way of the urine.

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BILE SALT METABOLISM.

IX. ECK FISTULA MODIFIES BILE SALT OUTPUT.

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It will be seen that an Eck fistula modifies the bile fistula output of bile salts and in several respects changes the reaction to standard procedures. The Eck fistula operation shunts the portal blood direct into the vena cava and limits the blood supply of the liver to the hepatic artery. The Eck fistula liver undergoes a certain amount of atrophy but with proper care there are no clinical disturbances and these dogs live a normal life for several months exactly as do the simple bile fistula animals. That the Eck fistula shows an output level of bile salt somewhat below that of a simple bile fistula is perhaps not surprizing but these observations like the chloroform experiments point to the liver cell as the tissue wholly responsible for production of bile salts in the body.

In an earlier paper (1) has been reported the bile salt output in an Eck fistula dog on a diet of cracker meal and beef heart. The average output of bile salt per 6 hours amounts to 14 mg. per kilo or 56 mg. per kilo per 24 hours, which corresponds closely to the figures given in the experiments below. This level is much below the bile salt output of other simple bile fistula controls under an identical régime as given in the same publication. These observations concern a series of open bile fistula dogs.

Methods.

General methods are exactly as described above and these experiments were carried along simultaneously with the simple bile fistula dogs. This Eck fistula operation has been described recently by Smith and Jones (2). The Eck fistula and closed sterile bile fistula are made at the same operation. The dogs tolerated

this operative procedure very well in spite of the long ether anesthesia. The clinical condition of both these dogs was excellent throughout the period of the observations tabulated below. The autopsy findings are given at the end of the experimental observations.

Experimental Observations.

The tabulated experiments given in this paper are to be compared with those of the preceding papers which give control levels. The ideal experiment would be very difficult and would consist of a series of observations on a simple closed bile fistula, after which an Eck fistula operation would give the Eck fistula output strictly controlled by the fore period in the same dog. The operative difficulties presented by this type of experiment are very great and have not as yet been surmounted. Different dogs have individual differences in bile salt output on salmon-bread or meat diets. These must be allowed for even if they cannot be satisfactorily explained. It may be only a functional difference analogous to obvious differences in muscular power in dogs as well as men.

Table 91 must be compared with Table 92 as the observations on this same dog are made at different periods, one shortly after the operation, the other 3 months after the operation. The clinical condition during this time was excellent and the bile remained sterile. The bile salt output on salmon-bread remains unchanged, showing 96 mg. per kilo in Table 91, and 103 mg. per kilo later in Table 92. There are differences in the bile salt levels for liver feeding which are clearly explained by the differences in liver intake in the diet. This dog did not relish cooked liver at first and would eat only small amounts. Beef muscle feeding shows a fairly good bile salt output of 157 mg. per kilo. There is a moderate cholagogue action.

The bile salt output shows a decided fall when *ferric citrate* is added to the salmon-bread ration. In this respect the simple bile fistula differs from the Eck-bile fistula (compare Table 84, Paper VIII above). How may we explain this difference between the simple and Eck-bile fistula dogs? More experiments of some variety are called for but it is suggested that this observation may indicate a diversion of amino acids which might have gone into bile salts but were used to form needed hemoglobin. The normal dog

or simple bile fistula dog in our animal colony shows a hemoglobin level of 130 to 150 per cent. This Eck-bile fistula dog showed

TABLE 91.

Liver, Steak, and Salmon-Bread Diet.

Dog 29-77.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg. .*	*mg.*	*mg.*
3–24		Salmon-bread 330				
25	13.0	" 440	156	0.32	50	1840
26	13.1	" 440	120	0.28	34	1250
27	13.3	" 440	116	0.33	38	1400
28	13.3	" 440	122	0.33	40	1470
29	13.1	" 440	108	0.41	44	1610
30	13.2	" 440	108	0.27	29	1060
31	13.2	" 440	112	0.25	28	1030
4–1	13.2	Liver 185	109	0.25	27	990
2	13.0	" 185	116	0.30	34	1250
3	12.5	Salmon-bread 220, liver 185	90	0.36	32	1170
4	12.6	" 220, " 185	140	0.29	41	1510
5	12.7	" 440	158	0.18	28	1030
6	13.1	" 440	140	0.22	31	1140
7	13.1	" 440	144	0.19	28	1030
8	13.1	" 440	150	0.19	29	1060
9	13.1	" 440	146	0.23	34	1250
10	13.0	Steak 380	128	0.27	34	1250
11	12.6	" 380	148	0.30	44	1610
12	12.6	" 380	166	0.35	58	2130
13	12.4	" 380	142	0.47	67	2460
14	11.8	Salmon-bread 390	78	0.56	44	1610
15	12.2	" 365	61	0.69	42	1540
16	12.3		80	0.28	22	810

Eck-bile fistula operation March 18. Bile sterile.
Bile salt average per 24 hours.
Salmon-bread = 96 mg. per kilo.
Liver + salmon-bread = 89 mg. per kilo.
Beef muscle = 157 mg. per kilo.

a hemoglobin level of 94 per cent during this period. To correct this anemia and to build needed hemoglobin some amino acids may have been used which otherwise would have gone into

bile salts.   It is possible that this is a peculiarity of the Eck fistula machine and not due to the anemia.   The amount of ferric citrate, 0.44 gm. daily, contains about 80 mg. of Fe as metal and is about double the dose of iron needed for anemic dogs to regenerate the maximum amount of new hemoglobin as defined elsewhere (3).

TABLE 92.

Liver, Salmon-Bread, and Ferric Citrate in Diet.

Dog 29-77.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
6–9		Salmon-bread 310				
10	12.5	" 455	119	0.22	26	950
11	12.6	Cooked liver 360	150	0.22	33	1210
12	12.0	" " 360	164	0.26	43	1580
13	11.9	" " 360	168	0.31	52	1910
14	11.7	" " 360	154	0.30	46	1690
15	11.7	Salmon-bread 560	186	0.31	58	2130
16	12.1	" 455	132	0.30	40	1470
17	12.6	" 510	132	0.26	34	1250
18	12.8	" 295	161	0.21	34	1250
19	12.2	" 475	130	0.30	39	1430
20	12.7	" 405 + ferric cit. 0.44	154	0.21	32	1170
21	12.6	" 295 + " " 0.44	160	0.18	29	1060
22	12.2	" 560 + " " 0.44	156	0.13	20	730
23	12.4	" 560 + " " 0.44	146	0.17	25	920
24	12.7	" 560	150	0.12	18	660
25	12.8	" 560	194	0.19	37	1360
26	12.7		210	0.19	40	1470

Eck-bile fistula operation March 18.
Dog slightly anemic, hemoglobin 94 per cent.
Bile salt average per 24 hours.
  Salmon-bread = 103 mg. per kilo.
  Liver = 154 mg. per kilo.
  Iron + salmon-bread = 68 mg. per kilo.

Table 93 shows a series of feeding periods on another Eck-bile fistula dog.   This dog also was in fine clinical condition and the bile was sterile.   It is noted that this dog is *distinctly subnormal in bile salt output*.   This is particularly noted with liberal liver and

beef muscle feeding where the output figures for bile salt are only 82 and 89 mg. per kilo per 24 hours. The bile salt output level for salmon-bread is also low, 70 mg. per kilo. The cholagogue action of these meat products is also subnormal.

TABLE 93.

Liver, Beefsteak, and Salmon-Bread Feeding.

Dog 29-14.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
11–4		Salmon-bread 365				
5	14.7	Pig liver 380	64	0.25	16	590
6	14.4	" " 250	94	0.27	25	920
7	14.4	" " 340	95	0.33	31	1140
8	14.3	" " 200	116	0.39	45	1650
9	14.0	Salmon-bread 440	41	0.67	27	990
10	14.2	" 420	64	0.48	31	1140
11	14.2	" 275	62	0.32	26	950
12	14.5	" 420	73	0.35	25	920
13	14.7	Beefsteak 260	69	0.31	21	770
14	14.3	" 195	104	0.28	29	1060
15	14.2	" 260	67	0.39	26	950
16	14.1	" 120	96	0.37	36	1320
17	14.1	Salmon-bread 440	99	0.39	38	1400
18	14.3	" 390	64	0.43	27	990
19	14.6	" 360	72	0.40	29	1060
20	14.8	" 440	80	0.38	30	1100
21	14.8	" 420	87	0.37	32	1170
22	14.7		74	0.35	26	950

Eck-bile fistula operation October 10. Bile culture negative throughout.
Bile salt average per 24 hours.
Salmon-bread = 70 mg. per kilo.
Beef muscle = 89 mg. per kilo.
Liver = 82 mg. per kilo.

Table 94 shows the bile salt figures for egg albumin and egg albumin plus glycine. These experiments are to be compared with similar ones with simple bile fistulas (see above, Paper VI, Tables 68 and 69). The Eck fistula presents distinct differences in this type of experiment. The simple bile fistula shows a bile salt out-

put level of 100 mg. per kilo for salmon-bread, 50 mg. per kilo for
egg albumin, and about 85 mg. per kilo for egg albumin plus glycine.
The Eck-bile fistula under identical conditions shows the bile salt
output levels as follows: for salmon-bread 80 mg. per kilo, for egg
albumin 81 mg. per kilo, and for egg albumin plus glycine 85 mg.

TABLE 94.

Egg Albumin Alone and with Glycine Feeding.

Dog 29-77.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
5–25		Salmon-bread 440				
26	12.8	" 370	155	0.19	30	1100
27	12.7	Egg albumin 110, cane-sugar 150	146	0.24	35	1290
28	12.5	" " 110, " 150	106	0.24	26	950
29	12.2	" " 110, " 150	90	0.34	31	1140
30	12.2	" " 110, " 150	72	0.33	24	880
31	12.3	Salmon-bread 530 ·	116	0.24	28	1030
6–1	12.8	" 560	142	0.17	24	880
2	13.2	" 560	159	0.14	22	810
3	13.4	Egg alb. 110, sugar 170, glycine 20	136	0.21	29	1060
4	13.3	" " 110, " 170, " 20	54	0.37	20	730
5	12.9	" " 110, " 170, " 20	38	0.97	37	1360
6	11.9	" " 110, " 170, " 20	67	0.45	30	1100
7	11.8	Salmon-bread 290	64	0.43	28	1030
8	12.3	" 450	113	0.21	24	880
9	12.7	" 310	120	0.26	31	1140
10	12.5		119	0.22	26	950

Eck-bile fistula operation March 18.   Bile sterile.
Bile salt average per 24 hours.
  Salmon-bread = 79 mg. per kilo average of all salmon-bread.
  Egg albumin = 82 mg. per kilo.
  Egg albumin + glycine = 85 mg. per kilo.

per kilo. Why the dog with Eck-bile fistula given egg albumin
should show a decidedly higher level of bile salt output than one
with simple bile fistula is not apparent.   The fact that glycine does
not supplement the egg albumin in the Eck-bile fistula, as we have
seen in the simple bile fistula, is intriguing.   Does this mean that
the dog with Eck fistula has a store of glycine at hand or that

glycine can be produced more readily under Eck fistula conditions?

The lack of cholagogue effect of egg albumin is obvious in these experiments (Table 94).

Table 95 shows two points of interest: that the steak digest gives conspicuously low bile salt figures and that the Eck fistula

TABLE 95.

Meat Digest and Bile Salt Feeding.

Dog 29-14.

Date.	Wt.	Diet.		Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro-cholic acid.
					In 1 cc. bile.	Total output.	
	kg.		gm.	cc.	mg.	mg.	mg.
1–14		Salmon-bread	240				
15		"	230	80	0.29	23	840
16	14.6	"	240	87	0.30	26	950
17	14.4	"	85, bile salt 6.9 gm.	89	0.27	24	880
18	13.5	"	440	126	0.70	88	3200
19	14.6	"	330	117	0.19	22	810
20	14.6	"	300	134	0.19	25	920
21	14.2	"	53	106	0.28	30	1100
22		"	400	54	0.34	18	660
23	14.6	"	240	68	0.31	21	770
24	14.2	*Digest* from 560 gm. steak		90	0.27	24	880
25	13.9	" " 560 " "		58	0.34	20	730
26	13.0	" " 560 " "		40	0.45	18	660
27	12.7	" " 560 " "		25	0.59	15	550
28	12.5	Salmon-bread	440	16	0.72	12	440
29	13.1	"	100	20	0.80	16	590
30	13.1	"	260	31	0.67	21	770
31	13.5			54	0.41	22	810

Eck-bile fistula operation October 10.   Bile sterile.
Bile salt average per 24 hours.
  Salmon-bread =. 60 mg. per kilo.
  Meat digest = 46 mg. per kilo.

liver cannot handle a moderate dose of bile salt by mouth without considerable loss in the turnover.

A meat digest was tested above (Paper V, Tables 57 and 58) and shows low output levels of bile salt which are equal to or a little below the salmon-bread diet levels of bile salt output.   In the Eck-bile fistula (Table 95) the bile salt output level for salmon-bread is

60 mg. per kilo and for meat digest is 46 mg. per kilo, which is close to the fasting level of endogenous bile salt output. This meat digest was prepared with less care to maintain neutrality during volume concentration of the original digest as described in Paper V. It is probable that some of the more important cyclic amino acids were destroyed and this may be responsible for the low bile salt output.

On the other hand the Eck fistula dog cannot tolerate as much meat feeding as the normal dog, especially a short time after the production of the fistula. This tolerance may increase a good deal as months pass. However this may have been a factor in this low output. We may compare whole beef feeding in this same dog at an earlier date where we see a higher level of bile salt production —89 mg. per kilo. There was no clinical evidence of intoxication in either experiment but we note a conspicuous loss of weight in the Eck-bile fistula. There is no cholagogue effect due to the meat digest although there is a moderate increase in bile flow due to whole meat feeding. This, too, indicates a destruction of some factors present in whole beef, presumably amino acids, destroyed by the digestion and concentration.

Table 95 shows another important point—that a single large dose of bile salt by mouth does not show approximate 100 per cent recovery in the next day's bile collection as is the case in the simple fistula (see Table 44, Paper IV of this series (4)). Bile salt, 6.9 gm., given as concentrated bile by stomach tube on January 17 is followed by a collection next day of 3.2 gm. of bile salt. In other words the Eck fistula liver recovers less than half what the normal liver would rescue from the gastrointestinal tract via the portal circulation.

Table 96 is to be compared with Table 97 as the experiments are much alike. We refer to Table 85 in Paper VIII for the control experiment with bile refeeding in a simple bile fistula to show the high plateau levels of bile salt output on salmon-bread (875 mg. per kilo) and on beef muscle (1470 mg. per kilo).

Bile refeeding (Table 96) is a simple procedure consisting of administration of 1 day's collection by stomach tube during the early part of the next day. The small amount of bile taken from the 24 hour collection for analysis is replaced by an equal amount of stock bile. This introduces a trivial error into the tabulated

figures under "bile fed." The maximum plateau level of bile salt production in this dog is 600 mg. per kilo which is much below the figures given in many other control experiments with simple bile fistulas, minimum 700 and maximum 875 mg. per kilo. The sal-

TABLE 96.

Bile Salt Output Levels with Bile Refeeding.

Dog 29-77.

Date.	Wt.	Diet.	Bile fed.		Bile secreted.			
			Vol.	Total taurocholic acid.	Vol.	Amino nitrogen.		Total taurocholic acid.
						In 1 cc. bile.	Total output.	
	kg.	*gm.*	*cc.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*
5–5		Salmon-bread 260						
6	12.2	" 260	158	3500	158	0.60	95	3500
7	12.2	" 240	169	4400	169	0.71	120	4400
8	12.2	" 170	186	4700	186	0.69	128	4700
9	12.1	" 240	160	4900	160	0.84	134	4900
10	12.0	" 270	219	5200	219	0.65	142	5200
11	12.0	" 180	212	5900	212	0.76	161	5900
12	11.9	" 450	224	5900	224	0.72	161	5900
13	12.3	" 300	272	6200	272	0.62	168	6200
14	12.2	" 410	326	7200	326	0.60	195	7200
15	12.3	" 420	324	6600	324	0.56	181	6600
16	12.4·	" 440	380	6800	380	0.49	185	6800
17	12.6	" 530	330	7500	330	0.62	204	7500
18	12.6	" ·400	329	7500	329	0.62	203	7500
19	12.3	" 260	281	7100	281	0.69	193	7100
20	12.5	" 450	241	7200	241	0.81	195	7200
21	12.8	" 370	300	7600	300	0.69	206	7600
22	12.5	" 420			282	0.64	180	6600
23	12.8	" 500			140	0.23	32	1170
24	12.9	" 560			144	0.24	34	1250
25	13.1				186	0.17	31	1140

Eck-bile fistula operation March 18.   Bile sterile throughout.

mon-bread level after this long period of bile refeeding is promptly reestablished at 92 mg. per kilo.

Table 97 shows a dog with a much lower plateau of bile salt production during bile refeeding on a salmon-bread diet. The maximum for this dog is 230 mg. of bile salt per kilo for 24 hours or

less than half that of the other Eck-bile fistula dog. This Eck-bile fistula shows a conspicuous difference in its capacity to handle a

TABLE 97.
Bile Salt Output with Bile Refeeding.

Dog 29-14.

Date.	Wt.	Diet.	Bile fed.		Bile secreted.			
			Vol.	Total taurocholic acid.	Vol.	Amino nitrogen.		Total taurocholic acid.
						In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	cc.	mg.	mg.	mg.
10–10		Operation						
11		Salmon-bread 0			29	0.91	26	950
12	15.0	" 175			8	0.22	2	73
13	14.8	" 160			90	0.32	28	1030
14	15.0	" 220			100	0.37	37	1360
15	14.7	" 250			82	0.37	30	1100
16	14.4	" 265			61	0.37	22	810
17	14.7	" 170			60	0.36	22	810
18	14.6	" 365	73	950	73	0.36	26	950
19	15.0	" 310	110	1800	110	0.44	49	1800
20	15.0	" 420	127	2460	127	0.53	67	2460
21	14.9	" 310	128	2710	128	0.58	74	2710
22	15.0	" 340	134	3050	134	0.62	83	3050
23	15.0	" 350	127	2970	127	0.64	81	2970
24	15.0	" 360	124	3020	124	0.66	82	3020
25	15.1	S.-bread 380 + bile salt 5.75	159	8170	119	0.60	71	2600
26	15.1	" 85 + " " 8.20	238	14200	198	0.83	164	6000
27	13.8	Salmon-bread 100	138	5300	138	1.05	145	5300
28	14.0	" 75	106	3300	106	0.84	89	3300
29	14.4	" 0			208	0.54	112	4100
30	14.4	" 160			204	0.70	143	5200
31	14.3	" 380			65	1.11	72	2640
11–1	14.9	" 325			76	0.39	30	1100
2	14.8	" 380			77	0.29	22	810
3	14.8	" 310			102	0.25	25	920
4	14.7	" 365			67	0.19	13	480
5	14.7				64	0.25	16	590

Eck-bile fistula operation October 10. Bile sterile throughout.

bile salt turnover as compared with any normal control during long periods of bile salt refeeding.

Table 97 also shows two considerable *supplementary feedings* given on October 25 and 26 to test the capacity of the dog to handle this moderate excess. This excess is largely lost and only a part of it appears in the next day's feeding, especially with the second dose of bile. The plateau level after this falls toward its previous level but bile refeeding was stopped a few days too soon to ascertain

TABLE 98.

Chloroform by Mouth or Inhalation Decreases Bile Salt Output.

Dog 29-77.

Date.	Wt.	Diet.		Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
					In 1 cc. bile.	Total output.	
	kg.		*gm.*	*cc.*	*mg.*	*mg.*	*mg.*
6–27		Salmon-bread	560				
28	12.8	"	560	188	0.20	38	1400
29	12.8	"	560	196	0.20	39	1430
30	13.0	"	560, chloroform 3 cc.	193	0.21	41	1500
7–1	13.2	"	560, " 3 "	134	0.20	27	990
2	13.1	"	560	98	0.04	4	147
3	13.4	"	560	130	0.08	10	367
4	13.3	"	560	220	0.15	33	1210
5	13.1	"	560	192	0.21	40	1470
6	13.1	"	560	172	0.21	36	1320
7	13.3	Fasting		210	0.18	38	1400
8	12.6	Salmon-bread	560, chloroform	136	0.26	35	1290
9	13.2	"	560	164	0.12	20	730
10	13.2	"	560	182	0.05	9	330
11	12.4	"	280	45	0.13	6	220
12		Dead in cage		28	0.12	3	110

Eck-bile fistula operation March 18. Bile sterile.
Chloroform in starch given by stomach tube June 30 and July 1.
Chloroform anesthesia for 1 hour July 8.
No clinical disturbance due to chloroform by mouth or by inhalation.

accurately what this final level would have been. The salmon-bread diet periods show the usual bile salt output when bile is not fed, 70 mg. per kilo.

Table 98 shows two experiments with chloroform which are to be compared with Tables 81 and 82 in Paper VIII. In the first experiment the chloroform is given by mouth on 2 successive days

and caused no clinical disturbance.   Moreover the food consumption is normal and the bile flow almost normal.   There is a definite fall in bile salt output to 10 to 20 per cent normal on the 2 days after the chloroform feeding.   This is an ideal experiment because the dosage was just right to cause slight liver injury but not enough to cause any notable fall in bile flow.   This excludes the possibility of biliary obstruction and indicates beyond a reasonable doubt that the disturbed function of the liver epithelium is responsible for the conspicuous drop in bile salt output.   Recovery is prompt and complete and the former level of bile salt output reestablished on the 4th day after the second dose of chloroform.

The second chloroform experiment was with an hour's chloroform anesthesia which probably caused a more definite liver injury (see autopsy below).   Unfortunately the dog developed a volvulus during the last 24 hours before death from shock.   The fall in bile salt output shows well in the 3 days following the chloroform anesthesia.

Autopsy.—Dog 29-77, Table 98.   The operative site and bile fistula tubing were all in good order with clean scar tissue.   Adhesions about the field of operation were responsible for a *volvulus* of the small intestine which is dark purple in color.   The mesentery is twisted and the vessels here are obviously obstructed.   The bile fistula is clean and bile ducts only very slightly dilated.   The Eck fistula is perfect and the portal vein completely obstructed at the hilus of the liver.   The liver is decreased in size.   The other organs present no abnormalities.

Histological Sections.—*Liver* shows normal periportal tissue and bile ducts.   There is no periportal cellular infiltration nor evidence of infection.   The liver lobules are small but in general normal except in the very central portion where occasionally are noted tiny hyaline central necroses, undoubtedly due to the chloroform anesthesia.   There is evidence of beginning liver repair with some phagocytes containing a little yellow pigment.   Intestines show the usual hemorrhagic necrosis due to the volvulus.   The other findings are not germane to these experiments.

Dog 29-14 (Table 95) some time after the last tabulated observations developed an obstruction in the rubber tube leading to the cannula in the common duct.   This complication is all too frequent in these closed bile fistulas.   After some weeks a false passage was

established between the obstructed common duct and the duodenum with the appearance of urobilin in the feces.   After an exploratory laparotomy the hepatic artery was ligated, causing death in a few hours.   The autopsy gave no information of interest for these experiments except the assurance that the Eck fistula was patent.

SUMMARY.

The Eck fistula may be combined with the simple closed sterile bile fistula.   Under these conditions we observe the following departures from the control experiments with a simple bile fistula.

A dog with *Eck fistula liver* shows a *subnormal* or even a great decrease in bile salt output on salmon-bread diets but especially on meat diets.

The dog with Eck fistula liver reacts entirely differently from one with control bile fistula liver to diets of egg albumin or egg albumin and glycine.

The dog with Eck fistula liver may react differently to iron salts with a decrease in bile salt output in contrast to one with the simple bile fistula.

The dog with *Eck fistula liver is incapable of turning over the usual amount of bile salt* whether given by refeeding of bile excreted or by the feeding of large supplementary doses of bile salt.   This may be due to change in the blood supply of the Eck fistula liver or to liver cell atrophy or both.

The dog with *Eck fistula liver shows a decrease in cholagogue action* due to various diets as well as bile salt feeding.

The dog with Eck fistula liver reacts to slight grades of chloroform poisoning as does the control dog with simple bile fistula. There is a great drop in bile salt output with no clinical disturbance and slight changes in bile flow.   Recovery of bile salt output parallels the well known reparative process within the liver lobule.

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OXIDATION-REDUCTION POTENTIALS OF CERTAIN SULFHYDRYL COMPOUNDS.

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The sulfhydryl systems have been shown by a number of investigators to be of fundamental importance in the respiratory interchange in tissues. Many attempts have been made to apply to them the methods of physical chemistry and especially those of electrochemistry in an endeavor more fully to elaborate the mechanism of the system. Dixon and Quastel (1) found that mixtures of cysteine and cystine and mixtures of the oxidized and reduced forms of glutathione could not be formulated in the usual electrochemical terms of reversible oxidation-reduction systems, in that the oxidized forms of these compounds had an insignificant effect on the potential at inert electrodes. They were able to calculate the observed potentials with the use of the following equation.

$$E = E_1 - \frac{RT}{F} \ln (\text{RSH}) + \frac{RT}{F} \ln (\text{H}^+) \qquad (1)$$

where E is the observed electromotive force, E_1 is an apparent reduction potential and the other symbols have their usual significance.

Kendall and Nord (2) studied the cysteine-cystine system in the presence of indigo carmine after treatment with molecular oxygen or hydrogen peroxide. They confirmed the observations of Dixon and Quastel and find evidence for an intermediate oxidized compound with which the reduced form is in equilibrium.

Michaelis (3) and his coworkers have made a very careful study of the sulfhydryl system and in a series of papers have developed the chemical and biological significance of this system. Michaelis and Flexner repeated the work of Dixon and Quastel and found

that they could calculate the potentials with a satisfactory degree of accuracy using Equation 1.   These investigators find that the system is very sensitive to traces of oxygen.   The technique they developed gave potentials reproducible within several millivolts. The results of the experimental work also indicated that the potentials observed by Dixon and Quastel were not accidental.

In a more recent study, Williams and Drissen (4) have attempted to treat the cysteine-cystine system by the ordinary titration method with the idea of determining the apparent oxidation potentials and have shown that this method has achieved some measure of applicability to this system.   It was suggested in this article that the observed potentials depended not only upon the presence of cysteine and hydrogen ions but also upon the presence of an intermediate oxidation product of the cysteine in the solution because curves having shapes similar to those of other oxidation-reduction systems were obtained.

Because of the importance of the sulfhydryl system in physiological processes and the interest in these results as an anomalous situation in electrochemistry, it was considered advisable to extend the study to other compounds.   The work to be reported in this paper deals with thioglycollic acid, $HSCH_2COOH$; monothioethylene glycol, $HSCH_2CH_2OH$; and α-hydroxy-β-sulfhydryl propionic acid, $HSCH_2CHOH \cdot COOH$.

<div align="center">EXPERIMENTAL.</div>

Procedure.

The apparatus used for the measurements was similar to that described by Clark and Cohen (5) and Michaelis and Flexner (3). The cell was a 2 inch weighing bottle and was arranged on a support together with the saturated calomel electrode.   The cell was immersed in a water thermostat which kept the temperature at $25° \pm 0.05°$.   All measurements were made at this temperature and in an atmosphere of nitrogen.   The gold and platinum electrodes were sealed into glass and inserted through the rubber stopper of the cell.   The whole arrangement facilitated the necessary manipulation.   Nitrogen was freed from the remaining traces of oxygen by passing it through a copper tube 110 cm. in length over finely divided copper, heated electrically to a temperature of

about 500°. The tube was packed with fine copper oxide wire which was subsequently reduced to the metal with hydrogen. The oxide formed during use was reduced at appropriate intervals. The nitrogen was bubbled through the solution at a slow and steady rate. All connections to the tube were brazed except the lead to the cell which was protected with a mercury seal.

The solutions used were prepared in standard buffer solutions. Solutions of the sulfhydryl compounds were added from a micro burette in the experiments in which the effect of concentration was studied. After each addition the potentials were read until the drift in electromotive force values was minimal and the potentials given by the gold and platinum electrodes agreed within several millivolts. Numerous titration experiments of the type reportéd by Williams and Drissen were also made. A Leeds and Northrup hydrogen ion potentiometer with enclosed scale galvanometer was used in this work.

The electrodes were of solid gold and platinum. As a routine procedure they were immersed in hot sulfuric acid-potassium dichromate mixture, then thoroughly rinsed with distilled water. Fairly good agreement of the measured potentials was obtained for the gold and platinum electrodes when the technique was rigorously standardized, though in some cases unexplained divergences occurred. Electrodes which gave erratic potentials were eliminated. The value of the saturated calomel electrode was taken as +0.246 volt at 25°.

The thioglycollic acid was an Eastman Kodak Company product. It was redistilled in a vacuum before use; boiling point, 104–106° at 11 mm. Hg pressure. The monothioethylene glycol was synthesized by the method of Bennett (6); boiling point, 69–70° at 28 mm. Hg pressure. The α-hydroxy-β-sulfhydryl propionic acid was prepared and purified by Koelsch (7).

Results.

The results of the experimental work will be described in two sections, as follows: "*(1) Variation of Potential with Concentration of Reduced Form and pH Value of Solution;*" "*(2) Electrometric Titration of Sulfhydryl Systems with Oxidizing Agents.*"

In the first section there has been studied for the several sulfhydryl compounds the validity of an equation, such as was used

by Dixon and Quastel, to express the relation between concentration of cysteine and the hydrogen ion concentration of the solution (Equation 1).

The second section is devoted to the treatment of these sulfhydryl compounds in solution by the ordinary methods of electrometric titration with the idea of determining the apparent oxidation potentials. In treating the systems in this manner it is necessary to assume a reversible reaction in order to derive the equation which is used to express the result.

1. Variation of Potential with Concentration of Reduced Form and pH Value of Solution.

In order to test Equation 1, given in the introduction, it is necessary to measure the electromotive force of a cell composed of a reference electrode (in this case a saturated calomel electrode) and an inert electrode which dips into a buffered solution of the reduced sulfhydryl compound. The concentration of the sulfhydryl compound and the pH value of the solution are altered at will. Typical data are presented in Table I, in which the pH values of the solution are given in Column 1, the concentrations of the reduced form are given in Column 2, the potentials at a gold and platinum electrode are given in Columns 3 and 4, respectively, and the values of E calculated from the relation of Dixon and Quastel, Equation 1, are given in Column 5.

The calculation for the values of E was made as follows:

$$E = -0.180 - 0.0591 \log (RSH) + 0.0591 \log (H^+)$$

The value of E_1 in the equation, -0.180, was an assumed constant. For monothioethylene glycol, at pH 3, concentration 0.00631 M,

$$E = -0.180 + 0.0591 (2.2) - 0.0591 (3)$$
$$E = -0.227 \text{ volt}$$
$$\text{Observed, } -0.225, -0.232$$

In general it may be said that the potentials are logarithmic functions of the concentration of the reduced form and the hydrogen ion concentration of the solution. From an inspection of Table I it will be seen that the calculated and the observed values show fairly good agreement except in concentrations ap-

proaching 0.1 M and where the pH of the buffer solution was above 7. This result may be explained in part at least on the basis of the acid dissociation of the hydrogen of the sulfhydryl group and the carboxyl group when the relative acidity was on the alkaline

TABLE I.

Potentials Measured against Saturated Calomel Cell.

Add 0.246 volt to convert to hydrogen electrode. All measurements at $25° \pm 0.05°$. $E_1 = -0.180$.

pH (1)	Concentration RSH. (2)	E_{Au} (3)	E_{Pt} (4)	E calculated. (5)
		Monothioethylene glycol.		
2.00	0.0015	−0.133	−0.136	−0.131
3.00	0.00068	−0.174	−0.165	−0.170
3.00	0.0153	−0.249	−0.240	−0.250
3.00	0.00631	−0.232	−0.225	−0.227
3.00	0.133	−0.285		−0.305
6.45	0.00496	−0.432	−0.432	−0.426
6.45	0.00984	−0.446	−0.446	−0.444
6.45	0.0654	−0.490	−0.490	−0.492
6.45	0.151	−0.513	−0.516	−0.513
8.15	0.00172	−0.507	−0.480	−0.499
8.15	0.0166	−0.548	−0.504	−0.558
		Thioglycollic acid.		
2.00	0.0017	−0.117	−0.116	−0.132
3.00	0.0031	−0.212	−0.215	−0.209
3.00	0.031	−0.263	−0.265	−0.267
6.45	0.00337	−0.400	−0.398	−0.416
6.45	0.00678	−0.420	−0.410	−0.433
8.15	0.000298	−0.455	−0.437	−0.455
8.15	0.00098	−0.487		−0.486
8.15	0.00328	−0.476		−0.514
8.15	0.00593	−0.460		−0.531

side of neutrality. The solutions of monothioethylene glycol gave more readily reproducible results.

Though there is a noticeable agreement between the observed and calculated values, it must be remembered that the constant E_1 is not a true normal oxidation potential, for the experiments

and calculations are made without reference to the oxidation product. The mechanism of the potential at inert electrodes given by only the reduced form is still obscure, and it appears that this approach to the problem cannot yield entirely satisfactory results. E_1 varies from experiment to experiment unless the technique is carefully standardized. This factor alone invalidates any extensive deductions from the magnitude of the value of E_1.
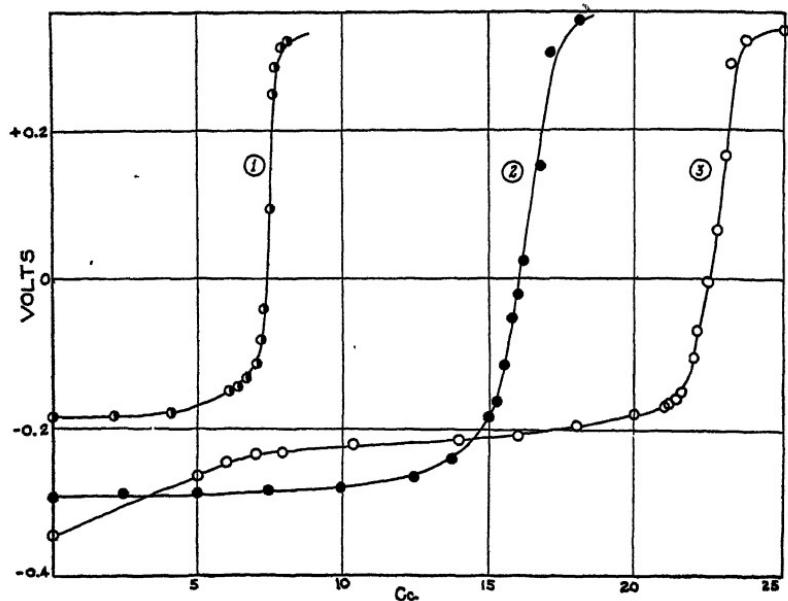
FIG. 1. Titration of sulfhydryl compounds with iodine. Curve 1, monothioethylene glycol at pH 5.3; Curve 2, thioglycollic acid at pH 6.5; Curve 3, thioglycollic acid at pH 5.8 (cf. Table II). Potentials are measured against a saturated calomel cell. Temperature, 25°.

2. Electrometric Titration of Sulfhydryl Systems with Oxidizing Agents.

Inasmuch as a method which neglects the effect of the presence of the oxidized component cannot yield results of theoretical value it was considered advisable to extend the method of electrometric titrations, previously used by Williams and Drissen for the cysteine-cystine system, to other sulfhydryl compounds, the

oxidized forms of which are entirely soluble at the concentrations employed. For such a procedure it will be necessary to assume a chemical reaction and derive the relation between the observed potential, the normal oxidation potential, and the concentration of the constituents. This has been previously done (4) so that only the final result will be given here.

$$E_h = E_0 + \frac{RT}{F} \ln (H^+) - \frac{RT}{F} \ln \frac{(RSH)}{\sqrt{(RSSR)}} \tag{2}$$

where E_h is the observed potential at the inert electrode, E_0 is the normal oxidation potential, and parentheses represent molecular concentrations.

For the oxidation, the solution of the sulfhydryl compound in a standard buffer solution was placed in the cell, and the cell was lowered into the thermostat. The oxidants used, iodine and potassium ferricyanide, were added from a burette in 1 cc. portions until the end-point, previously determined by a colorimetric titration, was approached when the titer was decreased. The two oxidants gave similar results, except that the potassium ferricyanide showed a slower attainment of equilibrium. Typical titration curves are shown in Fig. 1.

It was found possible to calculate the potential, E_0, at each stage of the oxidation by means of Equation 2. The results of a typical calculation are given in Table II. It is quite surprising how constant a value for E_0 is obtained, and it should be borne in mind that it is necessary to take into account not only the concentration of the reduced form, but that of the oxidized form as well in the equation used for this purpose. For each sulfhydryl compound at a given pH value it is possible to determine an E_0 value in this manner. The results of the experimental work have enabled us to calculate the E_0 for the several compounds and at several pH values. These data have been collected to form Table III. These values are for the most part averages of values calculated for several stages of the oxidation, as in Table II, but this procedure is not essential. The data may be studied by plotting first, potentials against cc. of oxidant, and, second $\frac{\Delta p}{\Delta cc.}$ against cc. of oxidant, where Δp is the increment in potential and

TABLE II.

Titration of Thioglycollic Acid (0.05 M) with Iodine Solution.

$T = 25° \pm 0.05°$.　pH $= 5.8 =$ constant throughout titration.

I₂ solution.	E_c	E_h	Oxidation.	$0.0591 \times \log \dfrac{(RSH)}{\sqrt{(RSSR)}}$	E_0
cc.			*per cent*		
0	−0.350		0		
5	−0.268	−0.022	21.9	+0.073	0.394
6	−0.251	−0.005	26.3	+0.068	0.406
7	−0.240	+0.006	30.7	+0.065	0.414
8	−0.237	+0.009	35.1	+0.061	0.413
10.4	−0.224	+0.022	45.6	+0.054	0.419
14	−0.217	+0.029	61.4	+0.041	0.413
16	−0.207	+0.039	70.1	+0.033	0.415
18	−0.195	+0.051	78.9	+0.022	0.416
20	−0.178	+0.068	87.8	+0.007	0.418
21	−0.166	+0.080	92.0	−0.005	0.418
21.1	−0.165	+0.081	92.5	−0.006	0.418
21.5	−0.159	+0.087	94.3	−0.013	0.417
21.7	−0.150	+0.096	95.1	−0.018	0.421
21.8	−0.133	+0.113	95.6	−0.021	0.435
22.8	−0.068		100.0		
Average..					0.416

TABLE III.

Summary of E_0 Values.

pH	E_0 HSCH₂CH₂OH.	E_0 HSCH₂COOH.	E_0 HSCH₂CHNH₂COOH.	E_0 HSCH₂CHOHCOOH.
2.0	0.35			
2.5		0.36	0.43	0.42
3.0	0.46	0.41, 0.42		
5.3	0.46	0.42		
5.8	0.44	0.42	0.44	0.42
6.5	0.44	0.42	0.44	
6.7	0.45			
7.3		0.42		
7.4	0.45			0.60
7.8		0.52		
8.2	0.45	0.66	0.58	

Δ cc. is the difference in titer. The point where $\dfrac{\Delta p}{\Delta cc.}$ reaches a maximum is the end-point of the reaction. Now the point of 90.5 per cent oxidation and 9.5 per cent reduction may be readily determined and the corresponding voltage read from the graph. At this point, the last term in Equation 2, $\dfrac{RT}{F} \ln \dfrac{(RSH)}{\sqrt{(RSSR)}}$ becomes equal to zero and if E_h is now expressed as E'_0, and for the temperature 25°, the equation may be written in the following form.

$$E'_0 = E_0 - 0.0591 \times \text{pH} \qquad (3)$$

In practise, then, to solve for E_0 it is only necessary to convert the potential read from the graph, E'_0, which is there referred to the saturated calomel cell, to the hydrogen electrode by adding 0.246 volt. To this is added the factor relating to the pH value of the solution.

In some cases the measurements were difficult to make because of a troublesome drift, but it was always less marked after the addition of the oxidizing solution. It amounted to as much as 20 or more millivolts in some cases. For this reason the final E_0 values given in Table III are reported to two significant figures only.

The reasonably satisfactory agreement for the values of E_0, calculated by making use of an assumed oxidation-reduction reaction and applying the laws of ordinary thermodynamics to it may be taken to indicate some fundamental reversible system between the reduced sulfhydryl compound and some intermediate oxidation product. This equilibrium determines the relative reduction intensity of the system. A number of investigators have made this suggestion from different, and in some cases unrelated, data (1, 2, 4, 8, 9). It appears from the work that the chief factor in determining the reducing level of the compounds studied is primarily that of the sulfhydryl group. At pH values above 7.4 and below 2.5 other factors enter, as is indicated by the divergence in the values for the normal oxidation potential in these regions. These may be directly related to the acid dissociation constants of the sulfhydryl and carboxyl groups of the compounds, but the data of the present article are inadequate to be of assistance in defining that relation precisely.

The values for the constant E_0 are in substantial agreement with those reported by Williams and Drissen for similar experiments. The magnitude of the potential, $E_0 = ca.$ 0.44, defines a reducing intensity or oxidation-reduction level approximately that predicted from studies by indicators; *viz.*, intermediate between indigo carmine and methylene blue. However it must be granted that at present there is little evidence that definite numerical values demarcate equally definite limits of oxidation-reduction levels. Other effects, conveniently classified as "specific chemical properties," undoubtedly play prominent parts. And it is partly for that reason that the more complete elaboration of the thermodynamical relations must await the compilation of further data.

There also emerges from this work additional evidence for the suggestion that the potentials observed in the study of the sulfhydryl systems depend upon the presence of some intermediate oxidation product as well as upon the presence of the reduced form.

The writer desires to acknowledge the help given by Professor J. W. Williams, under whose direction this investigation has been carried out.

SUMMARY.

1. The sulfhydryl compounds, thioglycollic acid, monothioethylene glycol, and α-hydroxy-β-sulfhydryl propionic acid, have been subjected to an electrochemical study.

2. It is shown that the observed potentials in the oxidation of these sulfhydryl compounds depend not only upon the presence of the reduced form and the hydrogen ion concentration of the solution, but also upon the presence of an oxidized form, presumably an intermediate of unknown chemical composition. The constancy of the calculated apparent normal oxidation potential indicates that the chief factor in determining the reducing level of the compounds studied is the sulfhydryl group.

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STUDIES ON THE CREATINE AND NITROGEN CONTENT OF THE WHOLE RAT AFTER THE FEEDING OF A VARIETY OF DIETS AND AFTER NEPHRECTOMY.

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The problem of creatine metabolism during high protein feeding has been excellently reviewed by Hunter (1) and Mitchell and Hamilton (2). A numbers of workers have suggested as a result of their experimental work that creatine is, in part, of exogenous origin (McCollum and Steenbock (3), Denis (4), and Mitchell, Nevens, and Kendall (5)). On the other hand Rose, Dimmitt, and Bartlett (6) believe "that no evidence has yet been adduced sufficient to justify the acceptance of a theory which postulates an exogenous origin of urinary creatine in the absence of creatine in the diet." Benedict and Osterberg (7) are inclined to question the significance of the creatinuria of forced protein feeding in connection with creatine metabolism.

Recent experiments of Bollman (8) seem to suggest a relationship between creatine-creatinine metabolism and a continued high level of protein feeding. Animals receiving daily intravenous injections of creatine did not show any increase in the creatinine output until comparatively large amounts of casein were fed over a period of 2 weeks.

Mitchell (2) concludes from his work (5) "that the concentration of creatine in the bodies of rats subsisting upon a protein-containing ration was distinctly higher than that of rats maintained for varying periods of time on a practically nitrogen-free ration." There was a marked difference of 16 per cent in the creatine concentration of these animals. Furthermore, the concentration of non-protein nitrogen, sulfur, and amino nitrogen was found to be unchanged. The conclusion is reached (5) "that tissue creatine is in part of exogenous origin, the concentration of exogenous creatine, representing an intermediary product in protein catabolism, depending primarily upon the intensity of catabolism of exogenous arginine and possibly of other creatine precursors."

If Mitchell's evidence and view-point are correct, we must assume that creatine metabolism in the organism is unstable and eas-

ily affected by many factors. Our own experimental work, however, has led us to conclude that the creatine concentration of tissues and the organism as a whole can be changed only under limited conditions of feeding and experimental manipulation. An extended study was therefore planned to investigate the effect of various diets on the creatine content of the eviscerated white rat. Additional studies were carried out on creatine-fed and nephrectomized animals.

TABLE I.

Composition of Diets Used.

	Diet 1.	Diet 2.		Diet 3.	Diet 4.
	per cent	*per cent*		*per cent*	*per cent*
Casein.*	25	75	Edestin. †	25	75
Dextrin..............	46	10	Dextrin..............	46	10
Lard.................	20	7	Lard.................	20	7
Cod liver oil..........	5	4	Cod liver oil..........	5	4
Salt mixture..........	4	4	Salt mixture..........	4	4

	Diet 5.	Diet 6.		Diet 7.		Diet 8.
	per cent	*per cent*		*per cent*		*per cent*
Gelatin. ‡	25	75	Dried extracted		Dextrin........	76
Dextrin........	46	10	beef..........	85	Butter fat......	10
Lard...........	20	7	Lard...........	7	Sucrose........	8
Cod liver oil....	5	4	Cod liver oil. ...	4	Salt mixture ..	3
Salt mixture....	4	4	Salt mixture....	4		

Osborne and Mendel's salt mixture was used. 200 mg. tablets of yeast concentrate (Harris) were fed daily.

* A commercial washed casein obtained from the Arthur H. Thomas Company of Philadelphia was used.

† A commercial product bought from the E. T. Pearson Corporation, Rockville Center, New York.

‡ Wilson's bacteriological gelatin.

EXPERIMENTAL.

All animals used in these experiments were healthy mature male and female rats. The stock animals were fed a prepared dog food powder[1] (29 per cent protein) on which they thrived and bred. The animals placed on the experimental diets were given food and

[1] Bal Ra, a dog food prepared by the Valentine Meat Juice Company, Richmond, Virginia.

water *ad libitum.* The control, high protein, and low protein diets are given in Table I. The basal diet for the 10 per cent creatine mixture consisted of $\frac{1}{3}$ whole milk powder, $\frac{2}{3}$ whole wheat flour, 1 per cent of the weight of the flour as NaCl, and 1 per cent of the weight of the flour as $CaCO_3$.

The rats were killed by a sharp blow on the back of the neck. The gastrointestinal tract was completely removed and discarded. It was felt that more accurate results could be obtained by the elimination of this tissue. The carcass was passed through a meat grinder three times and thoroughly mixed after each grinding. Aliquot portions of the material were immediately weighed and analytical procedures were begun at once.

Total nitrogen was determined by the usual Kjeldahl procedure. About 1 gm. of the ground tissue was used for analysis. Total solids were determined by heating 10 to 15 gm. of tissue for 48 hours at a temperature of 100–110°. This dried material was extracted with ether in a Sohxlet extraction apparatus for 4 hours for the estimation of fat. The ash content was obtained by incineration of about 10 gm. of tissue in a muffle furnace. Total creatine was determined by the method of Rose, Helmer, and Chanutin (9). Approximately 10 gm. of tissue were used for the determination of creatine. Duplicate analyses were made in the case of ash and creatine. No effort was made to separate the results obtained for males and females.

Analytical results obtained by analysis of a constituent in the carcass cannot be accurately gauged by the statistical method because of the many variables to be considered. The differences in the fat content alone can account for widely varying results obtained for creatine and nitrogen. Hence, it becomes necessary to eliminate as many variables as possible if a true picture of a constituent like creatine is to be obtained. In these calculations fat, ash, and water have been eliminated in order to attain this end.

The results of the experiments performed on rats fed on a variety of diets are recorded in Table II.

The large differences obtained for *fat* are attributable to the size and sex of the individual animals. There are no significant differences between the averages of the various groups. The average percentage concentration of *ash* remains quite constant

TABLE II.

Analyses of Rats on a Variety of Diets.

	No. of animals.	Weight of eviscerated animals.	Change in weight.	Time of feeding.	Ether extract.	Ash.	Total solids.	Total solids − fat and ash (organic).	Total N.	Total N in dry, fat- and ash-free tissue.	Total creatine.	Total creatine in dry, fat- and ash-free tissue.
		gm.	per cent	days								
					Gm. per 100 gm. tissue (per cent).							
Control group.												
Low..........		151			4.4	3.18	32.8		3.18	14.1	0.200	0.97
High..........		305			22.3	4.52	45.7		3.68	16.9	0.282	1.27
Average	39	224			13.4	3.81	38.4	21.2	3.37	15.9	0.234	1.10
Low protein.												
Low..........		134			9.3	3.37	36.3		2.90	15.1	0.208	1.02
High.........		283			25.2	4.59	47.7		3.54	16.6	0.244	1.17
Average	20	206	−9.5	14	16.3	3.93	40.7	20.5	3.16	15.5	0.225	1.10
85 per cent dried extracted beef.												
Low..........		195			8.9	3.52	34.6		3.18	14.6	0.225	1.08
High..........		309			18.3	5.14	43.2		3.73	19.3	0.272	1.27
Average	35	240		39	13.8	4.05	39.5	21.7	3.49	16.1	0.251	1.16
25 per cent edestin.												
Low..........		150			10.5	3.67	36.1		3.09	14.7	0.214	1.06
High..........		258			19.6	4.16	44.6		3.64	17.2	0.265	1.26
Average	15	206	−2.6	23	15.5	3.83	40.2	20.9	3.32	16.0	0.243	1.16
75 per cent edestin.												
Low..........		160			10.6	3.05	36.8		3.17	14.7	0.220	1.10
High....,.....		291			17.6	4.83	42.1		3.55	16.7	0.250	1.23
Average	13	228	−10.3	14	14.9	3.98	40.1	21.2	3.35	15.9	9.239	1.15
25 per cent casein.												
Low..........		143			12.6	3.60	37.6		3.00	14.8	0.228	1.08
High.........		279			20.5	4.17	43.5		3.32	16.4	0.247	1.21
Average	12	201	+5.6	17	17.1	3.88	41.2	20.2	3.16	15.7	0.234	1.16

TABLE II—*Concluded.*

No. of animals.	Weight of eviscerated animals.	Change in weight.	Time of feeding.	Ether extract.	Ash.	Total solids.	Total solids – fat and ash (organic).	Total N.	Total N in dry, fat- and ash-free tissue.	Total creatine.	Total creatine in dry, fat- and ash-free tissue.
						Gm. per 100 gm. tissue (per cent).					

75 per cent casein.

		gm.	*per cent*	*days*								
Low..........		150			7.7	3.70	33.4		3.40	15.6	0.240	1.13
High.........		235			12.5	4.60	38.8		3.74	17.2	0.268	1.26
Average......	9	175	−6.2	18	10.3	4.22	35.8	21.3	3.51	16.4	0.255	1.19

25 per cent gelatin.

Low..........		161			7.9	3.63	37.6		3.02	14.6	0.218	1.02
High.........		314			22.5	4.82	46.0		3.50	17.3	0.256	1.21
Average......	16	209	−8.2	14	14.2	4.20	39.5	21.1	3.24	15.3	0.236	1.11

75 per cent gelatin.

Low..........		140			5.4	4.04	33.0		3.24	14.4	0.222	1.02
High.........		287			12.3	5.23	42.0		3.77	16.5	0.253	1.11
Average......	16	189	−18.0	14	9.8	4.79	36.8	22.2	3.53	15.7	0.239	1.07

with the exception of the 75 per cent gelatin group, where we find an 18 per cent loss of weight. The changes in the percentage of *total solids* are negligible.

The individual *nitrogen* analyses show a marked variation, which may be due to the small quantity of material used. Nevertheless, the average results obtained for total nitrogen in the various groups do not vary as much as 0.4 per cent. The total nitrogen in the dry, fat- and ash-free tissue shows a maximum difference of 1.1 per cent between the various groups and a maximum deviation of 0.6 per cent from the control group. We feel that in view of the close agreement for the average per cent of nitrogen found in the carcasses of rats fed on a series of widely different diets, we may assume that the percentage of nitrogen in the organism remains unchanged.

Our experiments seem to indicate without a doubt that the creatine concentration of the organism remains constant under the

conditions of the experiments outlined above. The analyses were performed in duplicate on an adequate amount of tissue and the results obtained checked extremely well. The maximum individual variation for the per cent of creatine in the dry, fat- and ash-free tissue amounted to 0.3 per cent for a group of 175 animals. The results of the averages of individual groups justify the conclusion that the quantity and type of protein in the diet does not affect the creatine concentration of the organism.

Previous work (10) has shown that the creatine concentration of muscle and other tissues is slightly increased after complete nephrectomy in the white rat. A further study was undertaken of the creatine and nitrogen concentration of the whole organism 48

TABLE III.

Analyses of Rats 48 Hours after Nephrectomy.

	No. of animals.	Weight of eviscerated animals.	Change in weight.	Ether extract.	Ash.	Total solids.	Total solids — fat and ash (organic).	Total N.	Total N in dry, fat- and ash-free tissue.	Total creatine.	Total creatine in dry, fat- and ash-free tissue.
							Gm. per 100 gm. tissue (per cent).				
		gm.	per cent								
Low............		223		7.3	3.64	32.1		3.13	14.6	0.212	1.09
High...........		314		14.2	4.39	36.5		3.48	17.0	0.248	1.24
Average........	12	263	−2.0	11.6	4.01	35.9	20.3	3.29	16.3	0.236	1.16

hours after total nephrectomy. The results are given in Table III. It appears from this work that there is no change in the creatine or nitrogen concentration of the organism. The total solids decrease as is to be expected under these conditions.

Experiments were carried out to study the storage of creatine in the whole rat after feeding creatine for varying periods of time. In Table IV it is seen that the average results for fat, ash, and total solids obtained for all creatine-fed animals are practically identical with those of the control animals. The total creatine increases from a normal average value of 0.234 to 0.297 per cent; the creatine in the dry, fat- and ash-free tissue increases from a normal value of 1.10 to 1.41 per cent. It will be further noted

that the creatine concentration of the organism remains unchanged after the first day's feeding.

In connection with other experimental work data were obtained on four rats whose mother had been on an 85 per cent dried extracted

TABLE IV.

Analyses of Rats on a 10 Per Cent Creatine Diet.

	No. of animals.	Weight of eviscerated animals.	Duration of diet.	Ether extract.	Ash.	Total solids.	Total solids fat and ash (organic).	Total N.	Total N in dry, fat- and ash-free tissue.	Total creatine.	Total creatine in dry, fat- and ash-free tissue.
						Gm. per 100 gm. tissue (per cent).					
		gm.	*days*								
Low..............		150		8.5	3.06	36.4		2.92	14.4	0.267	1.24
High.............		353		18.7	5.07	42.2		3.50	16.5	0.315	1.53
Average..........	45	214		13.6	3.94	38.6	21.1	3.28	15.6	0.297	1.41
Average..........	10	241	1	12.5	3.96	37.8	21.3	3.41	16.0	0.291	1.36
"	16	234	4	14.8	3.86	38.9	20 2	3.24	15.5	0.295	1.42
"	6	152	5	11.6	4.36	37.4	21.4	3.27	15.1	0.316	1.47
"	3	.160	6	14.6	4.06	38.7	20.0	3.17	15.9	0.285	1.43
"	11	211	7	13.7	3.80	38.4	20.9	3.24	15.4	0.294	1.40

TABLE V.

Analyses of Rats Fed on a High Protein Diet during Entire Life.

Final weight.	Eviscerated weight.	Age.	Ether extract.	Ash.	Total solids.	Total solids fat and ash.	Total N.	Total N in dry, fat- and salt-free tissue.	Total creatine.	Total creatine in dry, fat- and salt-free tissue.
			Gm. per 100 gm. tissue (per cent).							
gm.	*gm.*	*days*								
180.4	166.0	153	15.70	4.14	39.8	20.0	3.18	15.9	0.234	1.17
188.5	175.0	153	19.90	4.28	43.7	19.5	3.06	15.7	0.235	1.20
145.3	134.3	153	17.30	4.00	40.3	19.0			0.230	1.21
149.5	136.5	153	20.20	3.79	42.5	18.5			0.230	1.24

beef diet (Table V) before and during the period of gestation and nursing. The experimental animals were placed on this diet at the time they were weaned and the diet was continued up to the

time of death. The results for creatine and nitrogen are within the normal range. These data further emphasize the fact that the length of feeding and the quantity of protein in the diet has no influence on the concentration of creatine in the organism.

<div align="center">DISCUSSION.</div>

The experiments recorded in this investigation seem to demonstrate the fact that creatine metabolism, as judged by tissue analyses, is unusually stable. It is felt that the use of large numbers of rats and the estimation of creatine and nitrogen in the "organic carcass" justifies the above conclusion.

There is a great deal of data in the literature dealing with the probable precursors of creatine. Arginine and glycine seem to be the outstanding compounds in this connection. The work of Rose and his students (10, 11) presents strong evidence against any relationship between arginine and creatine. The evidence in favor of glycine as a creatine precursor has recently been given by Brand and his coworkers (12, 13). An increased output of creatine was obtained in cases of pseudohypertrophic muscular dystrophy after the ingestion of glycine and gelatin, a protein rich in glycine. Only a slight increase in the urinary creatine was obtained after the feeding of edestin. It is interesting to note that both glycine and arginine are not essential for the growth of rats. In our own experiments, ingestion of diets containing a variety of proteins at high and low levels and of a protein-free diet has failed to induce any change in the creatine concentration of the organism. Our results must lead us to the conclusion that the creatine concentration of the normal adult rat cannot be affected by a surplus or deficiency of amino acids derived from exogenous protein in the diet. These results and conclusions are contrary to those of Mitchell, Nevens, and Kendall (5).

It must be remembered that the theory of creatine formation from exogenous protein "is drawn from young animals, or from those, like women or the subjects of muscular dystrophies, whose muscles approach in a sense the juvenile type. Even in these the extent to which creatine production can be increased by dietary protein appears to be limited" (1). The majority of the evidence obtained after the feeding of excessive quantities of protein to adults does not support the theory of an exogenous source of crea-

tine. Our own evidence does not support this theory. However, it is well known that feeding experiments conducted on normal animals in order to determine a creatine precursor by studying the urine or tissues appear to be futile. If small quantities of creatine are formed as a result of exogenous metabolism, one may assume that this creatine is metabolized in the same way that comparable amounts of ingested creatine would be handled.

The failure to obtain any increase in the percentage concentration of creatine after complete nephrectomy is rather significant. The blood picture in these animals gives evidence of an active creatine-creatinine metabolism (14). Assuming the continued formation of small quantities of creatine after the removal of the kidneys, one can explain the results obtained because of insufficient analytical accuracy or the organism is capable of destroying or utilizing this excess creatine.

The organism reaches a maximum saturation in its creatine content during the 1st day, provided there is sufficient creatine in the diet. If the rat metabolism is similar to that of other animals and man, one must assume a gradual metabolic "acclimatization" despite a creatine saturation. Under these conditions there must be an effective mechanism for the "destruction" of creatine. Data are obviously needed to determine the percentage of creatine that can be accounted for. The apparent ease with which the organism is able to metabolize excess creatine in the diet after a "saturation" has been reached again emphasizes the difficulty in obtaining any evidence of a creatine formation from creatine precursors in the normal animal.

CONCLUSIONS.

1. The ingestion of diets containing varying amounts of casein, gelatin, edestin, and dried extracted beef is not accompanied by any variation in the creatine and nitrogen concentration of the whole white rat. The results obtained with animals fed on a protein-free diet do not differ from those fed on the high protein diets.

2. Animals fed on a diet containing 10 per cent creatine become "saturated" with creatine after the 1st day. Subsequent feeding of this creatine diet has no effect on the creatine concentration of the organism.

3. The creatine and nitrogen concentration of the organism remains unchanged 48 hours after nephrectomy.

4. Our evidence does not support the theory of an exogenous formation of creatine from ingested or catabolized protein. The organism is capable of limiting the percentage of creatine in the tissues under a wide variety of dietary conditions. In all probability, tissue creatine is endogenous in origin and its concentration is determined by the metabolic and functional needs of the cell (Rose).

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